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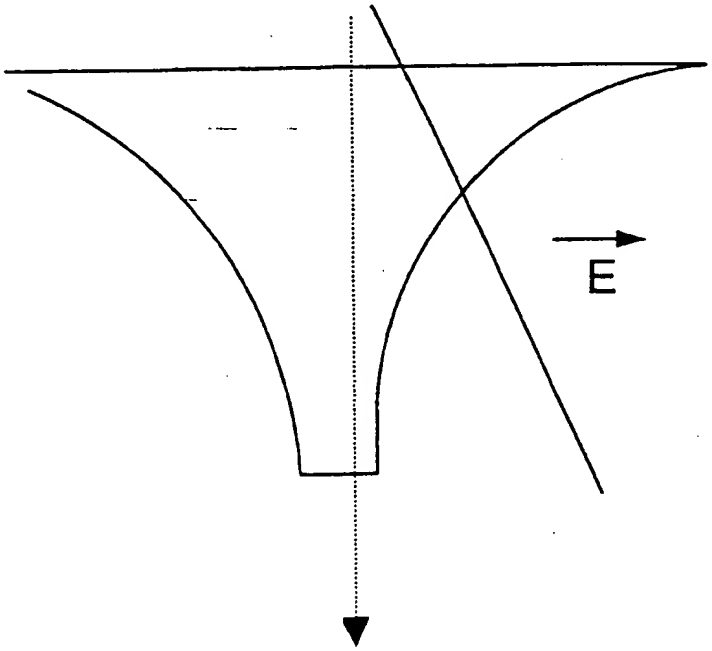

(57) Abstract: The invention concerns a method for moving, isolating and/or identifying particles in a sample by placing said sample in a spatially varied electrical field which acts independently and selectively on charged, dipolar and higher moments of the particle in a medium. The invention presented herein, comprises several novel methods of utilisation of the effect of nonlinear mobility and non-uniform electric field for preparative separation and improved analysis of macromolecules in a large variety of sizes and especially in the separation of DNA and its fragments and other biological molecules. Specifically, as demonstrated herein, the present invention uses nonlinear corrections of molecular mobility in an electric field in media, which are considered as important parameters for improved Separation and peak narrowing. The invention also provides an electrophoresis System which separates charged biological macromolecules by means of applying a non-uniform electrical potential across a buffer of electrolyte solution and supported gel, which contain those molecules. The system of the invention includes a power supply and control system which has a wide dynamic range of constant voltage, current and power which may be supplied, and is therefore particularly suited to the needs of the electrophoresis system.

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METHODS AND APPARATUS FOR NONLINEAR MOBILITY ELECTROPHORESIS SEPARATION

FIELD OF INVENTION

The present invention provides a method and apparatus for Separation and focusing of molecules, such as nucleic acids, using the effect of nonlinear mobility and of non-uniform electric fields.

BACKGROUND OF INVENTION

Electrophoresis (EP) dielectrophoresis and electrochromatography are widely used techniques for the analysis, purification, manipulation and separation of mixtures of macromolecules and the study of proteins and nucleic acids. Most applications of electrophoresis are based on the transport of molecules in a supporting gel medium, under the influence of a static electric potential and constant electric field. Many variations of constant-field electrophoresis have been employed based on tailoring the electric conductivity and field values by selecting the conductivity properties and pore sizes of the gels, the pH values and the electric potential magnitude. The most important in this category are one and two-dimensional electrophoresis, capillary electrophoresis, isoelectric focusing electrophoresis and others. Although most commonly used, these electrophoresis techniques have their limitations in separation capabilities regarding types of molecules, molecular sizes and molecular properties. In order to expand the capabilities and range of the techniques a large variety of methods and modifications have been developed for the electrophoretic separation of macromolecules and biological species. These new methods are based on the application of nonlinear molecular mobility in non-uniform electric

fields produced by change of the electric potential or by varying the electric properties of the separation media.

In 1984, Schwartz and Cantor described pulsed field gel electrophoresis (PFGE), introducing a new way to separate DNA. In particular, PFGE resolved extremely large DNA for the first time, raising the upper size limit of DNA separation in agarose from 30-50 kb to well over 10 Mb (10,000 kb). Applications of PFGE are numerous and diverse (Gemmill, 1991; Birren and Lai, 1990, 1993; and Van Daelen and Zabel, 1991).These indude doning large plant DNA using yeast artificial chromosomes (YAC's) (Ecker, 1990; see also Probe, Vol. 1, No. 1/2; and Butler, et al., 1992) and PI doning vectors (see Probe, Vol. 1, No. 3/4); identifying restriction fragment length polymorphism (RFLP's) and construction of physical maps; detecting in vivo chromosome breakage and degradation (Elia, et al., 1991); and determining the number and size of chromosomes (,,electrophoretic karyotype") from yeasts, fungi, and parasites such as Leishmania, Plasmodium, and Trypanosoma.

The simplest equipment is designed for Field Inversion Gel Electrophoresis (FIGE) (Carle, et al., 1986). FIGE works by periodically inverting the polarity of the electrodes during electrophoresis. Because FIGE subjects DNA to a 180^0 reorientation, the DNA spends a certain amount of time moving backwards. Only an electrical field switching module is needed; any standard vertical or horizontal gel box that has temperature control can be used to ran the gel. Although more complex in its approach, zero integrated field electrophoresis (ZIFE) (Turmel, et. al, 1990) also falls into this first category. Compared with simple FIGE, ZIFE is very slow. However, ZIFE is capable of resolving larger DNA and giving a larger useful portion of the gel. The other category contains instruments that reorient the DNA at smaller oblique angle, generally between 96^0 and 120^0 . This causes DNA to always move forward in a zigzag pattern down the gel.

A number of variants of pulsed-field gel electrophoresis (PFGE) have been described in the literature and are commercially available. In field-inversion gel electrophoresis (FIGE) the electric field alternates in polarity, and the

durations of the „forward" and „back" pulses (the pulse amplitudes) are chosen to achieve a particular separation; net migration is achieved by using a longer time or higher voltage in one direction than in the other. U.S. Pat. No. 4,737,252; Carle et al., Science, 232, 65 (1986). Several variants of field inversion gel electrophoresis (FIGE) have been described. In their original description of FIGE, Carle et al. presented separation data for identical field amplitudes, E.sub.+ =E.sub. -, but different forward and back pulse durations, t.sub.+ .noteq.t.sub. - (where E.sub.+ indicates an electric field causing a molecule to move away from its starting point in a gel, E.sub.- indicates an electric field causing a molecule to move toward its starting point in a gel, t. sub.+ indicates the duration of a single pulse in field E.sub.+ and t. sub.- indicates the duration of a single pulse in field E.sub.-). Carle et al. noted that resolution in a particular size range could also be achieved if t.sub.+ -4.sub.- but E.sub.+ .noteq.E.sub.-. Carle et al., Science, 232, 65 (1986). Somewhat better separations are possible if different durations are used for t.sub.~ and t.sub.-, and different amplitudes are used for E.sub. + and E.sub.- ; this method has been termed Asymmetric Voltage Field-Inversion Gel Electrophoresis (AVFIGE). Birren et al., Nucl. Acids. Res. 18, 1481(1990); Denko et al., Analyt. Biochem. 178, 172 (1989). A variant of AVFIGE, called Zero Integrated Field Electrophoresis (ZIFE) has been explored by Noolandi and Tunnel. Tunnel et al., in Electrophoresis of Large DNA Molecules, Birren and Lai (Eds.), Cold Spring Harbor Press, 101-132 (1990); Noolandi and Tunnel, Pulsed Field Gel Electrophoresis, in Methods in Molecular Biology, vol. 12, p. 73, Burmeister and Ulanovsky (Eds.), Humana Press (1992). In ZIFE, both the pulse times and the pulse amplitudes are varied during a run, while in principle maintaining the product (E.sub.+ t.Sub.+ equal to (E. sub.- t.sub.-). With this condition, .intg.Edt=0 over an integral number of cycles. A common feature of pulsed-field gel electrophoresis (PFGE) and its variants is that the time-dependence is the same in all areas of the gel. At any given time a single Set of parameters defines the electric field being applied to the gel, although those parameters may change during the course of the electrophoretic Separation. In contrast, in MZPFGE, multiple distinct electric fields are created

within the gel, with distinct spatial regions of the gel subjected to different fields at the same time.

Contour-clamped homogeneous electric field (CHEF) (Chu, et al., 1986, 1990); transverse alternating field electrophoresis (TAFE) (Gardiner, et al., 1986) and its relative ST/RIDEtm (Stratagene); and rotating gel electrophoresis (RGE) (Southern, et al., 1987; Anand and Southern, 1990; Gemmill, 1991; and Serwer and Dunn, 1990) are all examples of commonly used transverse angle reorientation techniques for which instrumentation is available. In a further elaboration of the above procedures, Lai and coworkers developed the programmable autonomously controlled electrophoresis (PACE) unit which allows complete control over reorientation angle, voltage, and switch time (Clark, et al., 1988; and Birren, et al., 1989). In contrast with FIGE, these Systems require both a special gel box with a specific electrode and gel geometry, and the associated electronic control for switching and programming the electrophoresis run.

TAFE and ST/RIDEtm use a complicated geometry between the electrodes and a vertically placed gel to get straight lanes. CHEF and RGE maintain a homogeneous electric field in combination with a horizontal gel. CHEF changes the direction of the electric field electronically to reorient the DNA by changing the polarity of an electrode array. With RGE the electric field is fixed; to move the DNA in a new direction, the gel simply rotates. Rotating Gel Electrophoresis (RGE) is one of the most recent commercial introductions of pulsed field equipment and combines variable angles with a homogeneous electric field (Southern, et al., 1987; Anand and Southern, 1990; Serwer and Dunn, 1990; and Gemmill, 1991).

Isoelectric focusing is an electrophoretic technique wherein an electric field is applied to a molecule in a pH gradient to mobilize the molecule to a position in the pH gradient at which its net charge is zero, i.e., the isoelectric point of the molecule. It often is used to separate proteins in a mixture and as an aid in characterization of biomolecules of unknown composition. Commercially available gradients may be utilized in isoelectric focusing which consist of

multicharged ampholytes, with closely spaced pI values and high conductivity, which partition into a pH gradient upon application of an electric field. The ampholytes are generally provided in a support matrix, such as a polyacrylamide gel. Molecules separated by isoelectric focusing may be visualized, e.g., by silver staining or Coomassie blue staining. Deutscher, Ed., Methods in Enzymology, Vol. 182, Academic Press, Inc., San Diego, Calif, 1990, Chapter 35.

Capillaries have been used in various electrophoretic techniques including isoelectric focusing. Novotny et al., Electrophoresis, 11:735-749 (1990). US. Pat. No. 5,061,361 (1991) relates to a capillary electrophoresis system in which a nanoliter volume of sample is introduced into the capillary tube, and an electric field is imposed on the system to effect separation of the charged components. After migration along the length of the tube, the sample components are detected via ultra-violet absorbance. U.S. Pat. No. 5,084,150 (1992) relates to an electrokinetic separation in which the surface of moving charged colloidal particles is treated so as to interact selectively with the sample molecules to be separated. An electric field is imposed on a capillary tube containing the colloidal particles and the sample to achieve separation. U.S. Pat. No. 5,045,172 (1991) relates to a capillary electrophoresis apparatus in which electrodes are attached at each end of a capillary tube, and a detector is coupled to the tube. U.S. Pat.. No. 4,181,589 (1980) relates to a method for separating biological cells using an electric field.

In electrophoretic methods for separating large double stranded DNA molecules, several techniques have been advanced to increase the band resolution (i.e., increase the distance between bands without a corresponding increase in the width of the bands, or decrease the width of the bands without a corresponding decrease in the distance between bands). The advantages of pulsing the electric field (i.e., periodically changing the field orientation) during gel electrophoresis of high molecular weight double-stranded DNA was first demonstrated by Schwartz and Cantor. Schwartz et al., Cold Spring Harbor Symp. Quant. Bi~. 47, 189 (1983); Schwartz and Cantor, Cell 37, 67 (1984); Cantor and Schwartz U.S. Pat. No. 4,473,452; Gardiner et al., Somatic Cell

Mol. Genet., 12, 185 (1986).

Further background information on conventional gel electrophoresis of DNA can be had by reference to a text such as Rickwood and Hames, Gel Electrophoresis of Nucleic Acids: A Practical Approach, IRL Press, Oxford, UK, particularly chapter 2, „Gel Electrophoresis of DNA", by Sealey and Southern. For background information on attempts to achieve Separation of very large DNA molecules by conventional gel electrophoresis, reference can be had to papers by Fangman, Nucleic Acids Res. 5:653-665 (1978); and Serwer, Biochemistry 19, 3001-3004 (1980). Implementation of the transverse-field technique (also defined as orthogonal-field-alternation gel electrophoresis, or OFAGE) and applications to the chromosomal DNA molecules from yeast are described by Carle and Olson, Nucleic Acids Res. 12: 5647-5664 (1984). A description of the complete analysis of the set of chromosomal DNA molecules from yeast using the transverse-field technique is further reported by Carle and Olson. Proc Natl Acad Sci (USA) 82: 3756-3760 (1985). Other background information on the application of the transverse- field technique of gel electrophoresis to chromosomal DNA molecules is provided by Van der Ploeg et al., Cell 37: 77-84 (1984); Van der Ploeg et al., Cell 39: 213-221(1984); and Van der Ploeg et al., Science 229: 658-661(1985).

The powerful impact of the isoelectric focusing method stimulates the search and development of new methods for molecular focusing and specially methods for DNA, chromosomes and cells focusing for which the IP (Isoelectric Point) method is unsuitable. In the last years the approaches were based on the choice of gels with optimal pore dimensions or varying pore size.

The careful study of the electric focusing process prompts the introduction of alternative detecting in vivo chromosome breakage and degradation (Elia, et al., 1991); and determining the number and size of chromosomes („electrophoretic karyotype") from yeast's, fungi, and parasites such as Leishmania, Plasmodium, and Trypanosome.

Various pulsed electrophoresis techniques have been proposed to improve the resolution of gel electrophoresis and expand the mass range of separated

molecules toward heavier and larger particles. Examples of these techniques are: „Orthogonal Field Alternating Gel Electrophoresis" (OFAGE) and Transversal Alternating Field Electrophoresis (TAFE) as proposed by K.Gardiner et al, Nature 331, page 371-2.(1988), „Field Inversion Gel Electrophoresis" (FIGE) described in US patent 4,737,251, and Zero Integrated Field Electrophoresis (ZIFE) as described by C.Turmel et al in Electrophoresis of Large DNA Molecules: Theory an Applications, Cold Spring Harbor Laboratory Press (1990). A specific application of FIGE for high resolution separation of single strand DNA was described by E.Lai in US Patent 5,178,737.

All these above listed techniques belong generally to the important method for separation and sorting of large particles and cells named dielectrophoresis and defined as the movement of a polarisable particle in a non-uniform electric field. The force arises from the interaction of the field non-uniformity with a field induced charge redistribution in the separated particle. This charge redistribution results in electrical polarization in the specific separation medium as expressed by the formula:

$$m = 4\pi\varepsilon_m K(\varepsilon^*_p, \varepsilon^*_m) a^3 E$$

where

$$K(\varepsilon^*_p, \varepsilon^*_m) = (\varepsilon^*_p - \varepsilon^*_m)/(\varepsilon^*_p + 2\varepsilon^*_m)$$

is the well known Clausius - Mossoti factor, ε^*_p and ε^*_m are the complex permittivities of the particle and medium respectively, a being the radius of the particle and E is the applied electric field. The medium generally applicable for these techniques is not limited to gels but can be any conductive liquid medium such as a buffer solution or electrolyte.

The basic equation, which determines the multipole force components acting on a dielectric particle in a nonhomogenous, axisymmetric electric field is given by:

$$F^{(n)}_z = 2\delta^\circ{}_1 K^{(n)} R^{2n+1} / n!(n-1)! \{?/?z [?^{n-1} E_z / ?z^{n-1}]$$

Here $F^{(n)}$ is the force component due to the n-th multipole interaction, K is the Clausius- Mossoti factor and E_z is the axial electric field.

Particles are manipulated using non uniform electric fields generated by various configurations of electrodes and electrode arrays. As a general biotechnological tool, dielectrophoresis is extremely powerful. From a measurement of the rate of movement of a particle the dielectric properties of the particles can be determined. More significantly, particles can be manipulated and positioned at will without physical contact, leading to new methods for separation technology.

A powerful extension of dielectrophoresis separation is the Traveling Wave Dielectrophoresis (TWD) in which variable electric fields are generated in a system of electrodes by applying time varying electric potential to consecutive electrodes.Such a method of Travelling Wave Field Migration was used by Parton et al. In US Patent 5653859.

A detailed explanation of the dielectrophoresis method was given by T.B.Jones in „Electromechanics of Particles", Cambridge University Press.1955.

A number of methods are known for cell separation. The mostly employed method is Flow Cytometry and FACS. Other methods include mechanical sorting, density gradient separation, magnetic sorting, electrostatic methods like field rotation sorting and dielectrophoresis.

The electrostatic methods when applied for cell handling have an inherent advantage in than other methods of manipulation like mechanical sorting, centrifugation, filtering or density gradient sorting. When the electrophoretic effect is used for the actuation of the cells, special precautions must be taken against electrolytic dissociation that might take place at the electrodes-solution interface. Dielectrophoresis is more suitable because the dissociation is avoidable with the use of high-frequency voltage.

Another important technique, which combines dielectric forces on particles with a gradient flow is the Field Flow Fractionation method. This method for the analysis and manipulation of mixtures of macromolecules, cells and chromosomes has been proposed and demonstrated in recent US patents 5858192 and 5888370 by Becker et all. Another similar technique applied to cell separation is the CFS (Charge Flow Separation) method as disclosed in US patent 5906724 and references therein.

A very important application of manipulation and design of electric fields is the field of microarrays and lab on chip system. In these systems microfluid channel arrays are combined with multi electrode arrays to produce a miniaturized laboratory for the movement, separation and identification of bioparticles. Example of such systems are as given by Manz et al in US Patent 5599432, Chow et al in US Patent 5800690, by J.M. Ramsey in US Patent 5858195, Zanzucchi et al in US Patent 5863708 and US Patent 5858 804. An interesting invention relating to microfluidic systems is given by Kopf-Sill et al in which variable dimension channels are introduced to facilitate the motion of particles through interconnects and turns (US Patent 5842787).

In all the above mentioned methods and inventions the design of the electric fields was obtained by manipulating the electric potentials in time and intensity and by addressing various electrodes.

Other methods of obtaining nonhomogenous fields have been based on introducing a concentration gradient in the separating medium or varying the thickness of the matrix material (Sugihara ,US Patent 5190629) and others by

utilizing gradient gels or by shaping the buffer layer thickness as proposed by D. Perlman in US Patent 5518604.

M.Washizu has proposed a system for manipulation of biological objects based on combination of electrodes and insulators in which variable field profiles could be obtained [M. Washizu, J. Electrostatics, V25,109-123 (1990)].

US-A-4,148,703 discloses a method of electrophoretic purification of enzymes and peptides, which is a continuous, modularized, one-step operation. The process is modularized with interchangeable parts and contains several divergent configurations of the electrodes, such as diagonal linear electrodes, point or ball electrodes, parabolic electrodes, arced electrodes and other geometrically shaped electrodes. The method enables the user to secure a high purification of enzymes and peptides simultaneously as it allows purification on a large scale. The modularization permits the easy insertion and removal different geometrically shaped electrodes, which allow a multifunctional versatile implementation and application of electrophoresis in the purification of electrically charged biomolecules. The method disclosed, however, is limited to charged biomolecules and relatively complicated to run.

US-A-4,261,835 deals with thin layered and paper chromatography and uses variable cross sections and shaped absorbent to control spreading of chromatography spots.

US-A-2,868,316 discloses conical, tapered separation columns for a kind of gas chromatography. Gas chromatography is not suitable to separate and purify biomolecules.

SUMMARY OF THE INVENTION

The invention concerns a method for moving, isolating and/or identifying particles in a sample by placing said sample in a spatially varied electrical field which acts independently and selectively on charged, dipolar and higher

moments of the particle in a medium.

The invention presented herein, comprises several novel methods of utilization of the effect of nonlinear mobility and non-uniform electric field for preparative separation and improved analysis of entities such as cells, particles, organelles, macromolecules in a large variety of sizes and especially in the separation of DNA and its fragments and other biological molecules such as RNA, lipids polysaccharides and the like. Specifically, as demonstrated herein, the present invention uses nonlinear corrections of molecular mobility in an electric field in media, which are considered as important parameters for improved Separation and peak narrowing.

The invention also provides an electrophoresis System which separates charged biological macromolecules by means of applying a non-uniform electrical potential across a buffer of electrolyte solution and supported gel, which contain those molecules. The system of the invention includes a power supply and control system which has a wide dynamic range of constant voltage, current and power which may be supplied, and is therefore particularly suited to the needs of the electrophoresis system.

BRIEF DESCRIPTION OF THE DRAWINGS

The features of the invention may best be understood by reference to the following description taken in conjunction with the accompanying drawings.

FIG. 1: Concentration of bromophenol blue dye in the electric field gradient under transition from region with a low conductivity determined by concentration of electrolyte buffer in amount of 0 1 TAE (Tris acetate EDTA buffer 0.004 M) to high electric conductivity (the buffer concentration is tris acetate EDTA buffer 0.04 M, pH =7.2) region. Panel A shows start of process; the dye is in start pocket; Panel B shows diffusion spreading of the dye during the electrophoresis; and Panel C shows the effect of dye focusing after crossing the boundary between the low and high conductive gels.

FIG. 2: Schematic of Hyperwedge with rectangular cross section. Electric field is linearly increasing in the X direction.

FIG. 3: Electropherogram of fragments of hydrolyzed DNA lambda phage HIND III in Hyperwedge 0.6% agarose gel. Voltage 20 V.

FIG. 4: Electropherogram of fragments of hydrolyzed DNA lambda-phage HIND III in 1.0% agarose gel. (Regular electrophoresis without wedge).

FIG. 5: The results of DNA lambda phage HIND III hydrolyzed fragments focusing. The field period is 138 seconds (reverse) +30 seconds (forward) and the non-homogeneous wedge parameter is 1:10. The experiment duration is 30 hours and operating voltages are -64 V and + 22 V respectively.

FIG. 6: Nonlinear electrophoretic mobility of fragments of DNA lambda phage HIND III hydrolizate.

FIG. 7: DNA lambda phage HIND III hydrolyzed fragments focusing results. The field period 138 (reverse) +30 (forward) seconds in a non homogeneous wedge parameter is 1:10. The experiment duration is 16 hours for the top picture and 36 hours for the bottom picture. Operating voltages are -64 V and + 22V.

FIG. 8: DNA lambda phage HIND III hydrolyzed fragments focusing results. The field period is 15 seconds (reverse) + 5 (forward) seconds in a non homogeneous wedge parameter of 1:10. The experiment duration is 30 hours and operating voltages are -62 V +22 V.

FIG. 9: DNA lambda phage HIND III hydrolyzed fragments focusing results. The field period is 15 (reverse) +5 (forward) seconds in a non homogeneous wedge parameter is 1:10. The experiment

longevity is 48 hours and operating voltages are -81 V +30 V with water cooling.

FIG. 10: DNA lambda phage HIND III hydrolyzed fragments focusing results. The field period is 15 (reverse) + 5 (forward) seconds in a non homogeneous wedge parameter of 1:10. The experiment duration is 24 hours and operating voltages are -81V + 30V with water cooling.

FIG. 11: 2-dimensional gel electrophoresis of DNA lambda phage HIND III hydrolyzed fragments. First dimension: nonlinear mobility drift during 24 hours (the field period is 15 reverse + 5 forward seconds and voltages are -81 V +27 V accordingly) with water cooling. Second dimension: regular electrophoresis with duration of 12 hours and voltage 15 V.

FIG. 12: Schematic diagram of the meander of electric field. E(t) = U(t)/L [V/cm]; E1 = U1/L [V/cm]; E2 = U2/L [V/cm]; T1 = [sec]; and T2= [sec] .

FIG. 13: Side view of an electrophoretic cell.

FIG. 14: Upper view of the electrophoretic cell having a hyperwedge gel geometry.

FIG. 15: Schematic of the geometric trap with linear coordinate dependence of the field strength $E(x) \sim \text{const-}x$.

FIG. 16: Schematic of the geometric trap with hyperbolic coordinate dependence of the field strength $E(x) \sim x^{-1}$.

FIG. 17: Schematic results of electrophoretic separation in the case regular electrophoresis for simple planar cell (A), and for hyperbolic geometric trap cell (B).

FIG. 18: Comparison results of separation of standard marker λ ladder by method of invention and conventional PFG separation. D. Conventional Pulsed Field Gel Electrophoresis to separate PFG λ ladder. Separation was achieved after 48.0 hours (up to ~

725.0 kb). E. and F. Methodology of the invention to separate PFG λ ladder. Preliminary experiments have achieved separation of over 550.0 kb in 3.5 hours.

FIG. 19: Comparison results of separation of standard low range PFG marker by conventional method and method of invention. Total run time to achieve separation of DNA up to 194.0 kb is 15 hours. Methodology of the invention to separate Low Range PFG Marker. Total run time to achieve separation of DNA above 242.0 kb is 2 hours. 2-D Pulsed Field Gel Electrophoresis of gel from Figure B. DNA fragments migrated according to size.

FIG. 20: Results of separation of several standard markers by method of invention. Lanes: 1. Low Range Marker; 2. lambda ladder; 3. s.cervese PFG marker 0.5 % Chromosomal Agarose in TAE; Forward Pulse: 88VX10 sec; Reverse Pulse: 36VX7sec; Total run time = 3.5 hrs.

FIG. 21: The non-uniform field in „start" method formed by concentration gradient of the buffer electrolyte. The geometry used in this method is a regular rectangular gel slab, as in regular electrophoresis.

FIG. 22: Velocity changes of DNA's fractions proportionally to the intensity of the field.

FIG. 23: Circuit schematic for power relay device

FIG. 24: Fraction's compression in linear wedge at constant potential

FIG. 25: Fraction's compression in hyperbolic wedge at constant potential

FIG. 26: General diagram of cell's separation system

FIG. 27: Separation chamber for cells

FIG. 28: Example of hyperbolic chamber for cell's separation.

FIG. 29: Double triangular wedge for cell's separation.

FIG. 30(a-o): Examples of variable cross-section separation chambers

FIG. 31: System for fast extraction of separated fractions. System includes special channel for extraction.

FIG. 32: Schematic shape of traveling concentration wave (dependence of concentration on coordinate)

FIG. 33: Type of electric signal for traveling concentration wave separation.

FIG. 34: Separation cell for traveling concentration wave separation.

FIG. 35: Result of traveling concentration wave separation of gonococcus bacteria. (+-50V)

FIG. 36: Traveling concentration wave separation of RBC.

FIG. 37: Separation of Monoclonaly Antibody. (5μg). A. Separation by moving boundary electrophoresis on either gels according to the invention (left panel) or regular (right panel) gel systems. Electrophoresis was preformed under 4W and 3W for gel according to the invention and regular gels, respectively. Gel (upper gel 3 % T, lower gel 7.5 % T) and anolyte included 10 mM Imidazole-HCl, pH 7. 75 mM boric acid-KOH, pH 9.23 was used as the catholyte.

FIG. 38: Moving boundary method separation. Separation by discontinuous polyacrylamide gel electrophoresis on either gels of the invention (left panel) or regular (right panel) gel systems. Electrophoreses was preformed under constant power, 4W for gels of the invention and 3W for regular gels. Running and gel buffers included 10 mM imidazole-glycine, pH 7.

FIG. 39: Moving boundary electrophoresis was used to separate human plasma. A comparison between rectangular and wedge shaped geometry under similar electrophoresis conditions shows that

the protein bands in the shaped wedge are more distinct and more proteins can be visualized. Separation of human plasma. Separation by moving boundary electrophoresis on either gel of the invention (left panel) or regular (right panel) gel systems. Electrophoresis was preformed under 4W and 3W for gels of the invention and regular gels, respectively. Upper gel (3 % T) included 15 mM Imidazole-HCl, pH 7 lower gel (7.5 % T) and anolyte were made with 15 mM Imidazole-0.1 M boric acid, pH 7. 0.1 M Boric acid-KOH, pH 9.23 was used as the catholyte. Time of electrophoresis was determined so proteins will migrate to similar positions in the two gels.

FIG. 40: RBC concentrate near the "hole", when a variable signal is applied.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for a new electrophoretic and dielectrophoretic separation technique and system for improved separation and manipulation of bio particles in an extended range of sizes and shapes. More specifically particles can be separated according to their mass, density, internal and surface charge distribution, shape and dielectric properties.

The invention provides for a new electrophoresis technique by applying a higher order manipulation of the electric field to enhance the resolution of separation of biomolecules, such as DNA and to extend the sizes of the biomolecules such as DNA, which can be separated. In other embodiments this invention provides for methods of separation and sorting of biomolecules such as proteins, chromosomes, cells and cell fragments.

According to the invention the method for moving, isolating and/or identifying particles in a sample by placing said sample in a spatially varied electrical field wherein the spatially varied electrical field is following a mathematical function selected from the group consisting of hyperbolic, parabolic functions and

combinations thereof. According to the invention also inverse functions such as $y \sim x^{-1/2}$.

In a preferred embodiment of the invention the spatially varied electrical field is achieved by varying the cross-section area of the medium normal to the direction of the electrical field.

In particular the mathematical functions are following combinations of hyperbolic, parabolic functions obtained by linear combinations, multiplications and/or divisions of said functions.

Preferably, the medium is limited by limitation means having a shape of two converging hyperbolas.

Based on electric current conservation law the local electric field at each point along the separation medium is proportional to the cross section area of the medium a multitude of spatially varied electric fields can be generated by designing the shape of the medium or medium enclosing vessel. The electric field profile is obtained between at least two electrodes placed at the ends of the separation vessel and in electrical contact with the separation medium by applying a voltage to the electrodes. Preferably at least two separation media having different dielectric or conductivity properties are combined according to the invention.

By this method either in a constant cross section vessel or medium or variable cross section vessel a desired electric field distribution is obtained by applying a voltage to electrodes at contact with the medium.

In another preferred embodiment of the invention the spatially varied electrical field is achieved by a multielectrode arrangement and the potential between two adjacent electrodes along the medium is different.

By this method a desired field (shape) distribution is obtained either in a constant cross section vessel or medium or variable cross section medium or vessel, in a single medium or multiple media, and this by inserting into the medium at least one additional electrode along the medium and supplying separate voltages to each electrode pair.

In particular the medium comprises a fluidum such as a gel, a liquid, a solid matrix. Each of these selected by their electric conductivity and dielectric properties and applicable for the generation of a particular electric field pattern and particular separation procedure as desired.

Preferably, the electric field is designed (changed) in a stepwise form from high field to low field to obtain initial focusing and compression of the fractions, This by causing the advancing (particles) molecules in the fraction to slow down when crossing the field step while the trailing particles are moving fast in the high field region.

According to the invention a steep field jump (drop) is preferred in the transition region between two gel segments, with different electrical properties. By joining two types of gels or other separation media possessing different conductivity properties and applying a voltage perpendicularly to the boarder line between the two gels a desired field step is produced.

The method of the invention allows for separating larger particles from smaller particles when dispersed in a medium by applying a non-linear electric field across the medium,

a) such that different particles will move in opposite directions

or

b) large particles move faster than small particles or vice versa.

The method of the invention allows for separation of particles according to their electrical properties such that particles of identical or same size but different consistency e.g. structure, conformation and the like, both for different size particles and same size particles For separating different particles, the method of the invention preferably comprises the following steps:

- suspending the particles to be separated in medium such as a gel or other conductive medium, wherein the medium has a variable cross section geometry,

- generating and applying a non linear electric fields across or along the medium causing the particles to travel through the medium according to their electrical properties in the medium.

It can be advantageous to vary the electrical field in a time dependent manner, e.g. periodically for focussing, i.e. immobilization of specific particles in the separation medium at a fixed location along the medium. By this embodiment of the invention it is possible to separate specific fractions of particles at predetermined position along the separation media for further analysis or processing.

Preferably the spatially varied electrical field is achieved by producing a complex separation sequence and media by generating a continuous path of a medium such as a separation gel made up from segments with different electrical properties and/or cross section area and by applying different electric potentials to each segment or by generating different electric fields in each segment. This is achieved either by placing the separation media such as a gel between two metal electrodes inserted at both ends of the media and by applying a constant or time varying voltage or by placing metal electrodes in each of the segments and supplying different voltages to each electrode.

In a further preferred embodiment of the invention the non uniform electric field is generated in a capillary.

In particular, the capillary is filled with segments of separation media such as gels with different electric properties.

By this method a field pattern consisting of regions with different field intensity is created upon applying an electric voltage along the capillary and by this way improved manipulation and separation can be achieved like fraction narrowing, lowering the operational voltage and shortening the separation length. The non-uniform field may be generated by segments of polymer tubes or inserts with different diameter or varying internal diameter placed in the capillary. By this way of designing the shape of the internal surface of the insert a variety of nonlinear field patterns are obtained along the capillary, these fields enabling

separation and transport of particles for the elucidation of particular electrical properties of the specific particle.

Another embodiment of this invention consists of a capillary produced with a variable cross section to obtain a non-uniform electric field distribution in the separation media along the capillary. Advantageously, the non polymerized gel is applied to the surface of the substrate by means of a special pen-like dispenser filled with gel, the pen being a part of a plotter operated by a special software. By this embodiment of the invention capillary like segments are produced having varying cross section area(s) and which are shaped to obtain a particular field distribution along the segment.

The gel sequence may also be assembled from segments of gel cut to a special shape for example by a software driven cutting machine.

The capillary sized segments can be also produced by machining shaped channels in a suitable substrate like glass, PMMA or others by a known machining method like etching or laser machining or others. By inserting metal electrodes to the ends of these segments and applying voltage from a power supply a particular separation procedure is obtained. By combining at least two different segments and by applying a multiplicity of voltages to different segments a complex separation and manipulation method can be designed. Preferably, the medium for separation according to the invention is arranged on a chip to form a microfluidic system and capillary electrophoresis and dielectrophoresis in which several segments filled with at least one separation medium are combined and by applying voltages to each segment complex manipulation and reaction procedures are conducted and detected.

Subject matter of the present invention is also a separation medium having a first end portion and a second end portion and a continually varying cross-section wherein the first end portion has a larger cross-section than the second end portion.

The separation medium of the invention comprises a continually varying cross-section according to a hyperbolic function.

The separation medium preferably is arranged in a means for supporting the medium such as glass plates, wedges, and the like.

A further embodiment of the present invention concerns a capillary having a first end portion and a second end portion and a continually varying cross-section wherein the first end portion has a larger cross-section than the second end portion to form a tapered capillary.

Also in this embodiment it is preferred that the capillary comprises a continually varying cross-section according to a hyperbolic function.

The separation media of the invention can be combined in order to form an assembly of at least two capillaries, wherein the second end portion of a first capillary is directed towards the first end portion of a second capillary.

Preferably, in this assembly the at least two capillaries are tightly joined to each other.

The capillary of the invention or the assembly of the invention my be filled with a separation medium such as a gel.

The capillary of the invention or the assembly of the invention are preferably manufactured of inert material such as glass or artificial resins such as polymethylmethacrylates PMMA and the like.

The present invention further pertains to an electrophoresis apparatus for performing the method of the invention. The apparatus of the invention is comprising a first electrode means comprising a cathode and an anode, means for supplying a non-uniform and time dependent, in particular periodical, electric field, and a separation medium preferably a separation medium of the invention, such as a gel, means for supporting the medium in a geometry of varying cross-section during use of the apparatus, the anode and cathode are disposed at respective opposed edges of the separation medium to produce a polarity electric field in the plane of the separation medium.

The apparatus of comprises means for applying designed electrical fields to the electrode means.

The apparatus comprises in particular additionally at least one reference electrode.

Further advantageous embodiments are achieved by combining the method of the invention with other electrophoretic methods, such as the temperature gradient electrophoresis.

The electric current flowing through a variable cross section cell filled with separation medium will cause non- homogenous Joule heating along the separation chamber and temperature gradients will be generated. These temperature gradients together with the non homogenous electric field can be utilised for further inventive embodiments like for example the separation of proteins by isoelectric focussing in a pH gradient.

For example a rectangular cross section separation vessel with the outer boarders shaped as a hyperbola will generate a linear electric field variation and a parabolic temperature dependence along the separation vessel. Such a temperature dependence will create a parabolic pH variation and will enable the separation of proteins in the pH interval, and vice-versa.

In the following more specific embodiments of the invention are described by virtue of some novel separation techniques rendered possible by the method of the invention

Separation of DNA

In a preferred embodiment of the present invention biomolecules such as DNA can be separated. In the following the method for separating DNA will be illustrated in greater detail.

The present invention provides for a new electrophoresis technique by applying a higher order manipulation of the electric field to enhance the resolution of DNA and to extend the sizes of DNA, which can be separated. As demonstrated herein, the data shows that a single DNA molecule moved in the

electric field in a very sharp (Fig. 6) focused band relative to a control (Fig. 4), thus clearly improving the resolution power and that the separation and resolving power of the technique with a collection of various size pieces of DNA. The present invention provides methods and an apparatus for the separation and manipulation of molecule which comprise the following: 1) virtual traps; 2) generation of steep field by gel composition for inverse focusing; 3) geometric traps and 4) large fragments separation.

The present invention provides electrophoresis apparatus comprising first electrode means consisting of a cathode and an anode and means for supplying a non-uniform and time dependent (periodical) electric field, and gel retaining means suitable, in use, to retain a gel in a wedge geometry within said apparatus such that, in use, the said anode and said cathode are disposed at respective opposed edges of said retained gel to produce a polarity electric field in the plane of said retained gel.

As demonstrated herein, the results show that two main methods of focusing in non uniform field were: the method of focusing in a continuous field gradient; and the method of concentration dependent approach or „Start" method. The concentration dependent approach or „Start" method comprises the generation of a steep field jump by proper selection of the electrical properties of two kinds of gels. The field jump when positioned at the start position of the electrophoresis cell causes a focusing (compression) of the fractions in the solution under analysis (Fig. 1).

In this experiment two segments of gel with different buffer concentration were used (TAE buffer with 10x higher concentration in one segments than in the second segment). This resulted in a conductivity ratio and consequently in a field ratio of 10x.

The concentration dependent approach is based on the principle that local changes in the conductivity of the gel will result in changes in the electric field. For example, if the supporting media (gel) consists of two sequentially located segments with different conductivity a non uniform electric field is formed on the segment boundary, when an electric potential is applied. By this means, by

using gel segments prepared on the basis of buffers with different concentration of electrolyte, different configurations of non uniform electric field may be formed. In doing so, standard buffer solutions can be applied near the electrodes. Since the dimensions of the transition region will be small in comparison with the length of the gel segments, the blurring caused by non uniform electrolyte concentration in gel, which is determined by diffusion, will be negligible. This method is useful for improved focusing and resolution in DNA separation.

The non-uniform field in „start" method formed by concentration gradient of the buffer electrolyte. The concentration in the „start" region is about 10 times smaller then for work region (0.004 TAE and 0.04 TAE accordingly). Because the conductivity of gel is defined only by concentration of the buffer electrolyte, the conductivity of the „start" region will be 10 times smaller than in other part of the gel. Since the total current does not change when it flows through the gel, as a result the electric field in the start region will be 10 times larger than in the other part of the gel. The geometry uses in this method is a regular rectangular gel slab, as in usual regular electrophoresis, as shown in Fig. 21.

2-electrodes(Pt) cell: length:23 sm, width:10 sm. Buffer 0.04M (0.004M) tris-acetate (SIGMA, Inc.)+0.002EDTA (SIGMA, Inc.) . Dye: Ethydium bromide (SIGMA, Inc.). Agarose (BioRad, mr=0.1). Voltage range: 20-150V.

In a continuous field gradient a strong longitudinal, nonuniform electric field is formed by variable geometry or by a concentration gradient method through the whole length of the gel. The field in wide part of a wedge is much smaller then in narrow one. For example, if the separation gel is in the form of a wedge than the non-uniform electric field along the wedge will obtain the wedge-like dependence. The method can be of interest in applications in the electrophoretic separation of fractions of very different in size.

The subsequent experiments provided herein, consist of methods of generation and design of electric field gradients for focusing in non-uniform field. The development was completed with the experimental verification of the

method in experiments on the focusing of fractions of fragments of the DNA marker: LAMBDA- HIND III hydrolizyte. In the case of non-uniform electric field the velocity changes proportionally to the intensity of the field. For example, see the Fig. 22. The corresponding values of nonlinear mobility of Lambda –Hind fragments are presented in Fig.8.

Virtual Traps:

The invention provides apparatus and methods for electrophoretic separation of macromolecules based on nonlinear mobility effects, which can be incorporated in current methods of electric separation: electrophoresis, electro chromatography. The invention simplifies the process of electrophoretic separation and enables separation without mechanical and chemical structuring of pH gradients in media (gels etc.) but by manipulation with non uniform electric fields.

The nonlinear mobility electrophoresis in non-uniform and time dependent (periodical) electric field is used for creating virtual traps for particles, like in the method of isoelectrical point (IP) focusing but without pH gradients.

This invention provides an apparatus having a special designed electric signal applied to the electrodes of the separation cell. The time dependent electrical voltage V(t) (meander) is presented in Fig. 12.

The total duration of one period of meander is $T=T_1+T_2$, and the average voltage is $\langle V\rangle=(V_1T_1-V_2T_2)/(T_1+T_2)$. The power supply gives Voltage V_1 and V_2 up to 210V with maximum current 100mA and provide time intervals from 400 sec to 1msec. Such PC-driven and electrically controlled electrophoretic device produces signals which must create virtual traps for the macromolecules in gel and have a number of advantages in comparison with the regular electrophoretic techniques. The advantages being higher resolution, higher sensitivity and important ability of focusing the particles, which have the nonlinear mobility. The nonlinear effect is closely connected with the secondary structure of macromolecules. As a result the nonlinear mobility focusing gives us an instrument for the investigations of secondary structure of macromolecules. A comparison of the regular electrophoresis and nonlinear

electrophoresis is presented in the 2 dimensional gel electrophoresis as presented in Fig. 11.

Coefficient of nonlinear mobility include dipole and quadrupole moments. By accounting for these coefficients special „traps" can be designed whereby the separation only according to dipole moment or only according to quadrupole moment is possible.

Special types of electrophoresis cells with two working electrodes and two reference electrodes have been constructed for the generation of non-uniform electric fields, which result in the nonlinear mobility focusing. Analytical complex functions depend on the shape of the electrophoretic cells. As provided herein as examples, two forms of gel slab have been considered: the simple planar wedge and the hyperbolic wedge (Hyperwedge). The parameters of a wedge (length L and the start and end crossection H1 and H2, in couple with voltage parameters V_1 T_1 V_2 T_2 are very important for the success of nonlinear mobility focusing. As an example in the case of zero average voltage $<V>=0$, all fraction are focusing in the same point, corresponding to maximum crossection of the wedge. Also if the average voltage $<V>$ is greater than a critical value, nothing focusing will be appear in the gel region.

GENERAL PRINCIPLE FOR ELECTRIC SIGNAL (MEANDER) (Fig. 12):

1) Assymetric square voltage waveforms $T_1 \neq T_2$ and $V_1 \neq V_2$

2) Choice of optimum values for V_1T_1 and V_2T_2

3) Gradient of electric field

4) The important condition for our method using for separation is use of power supply with stabilization on current and voltage (potentiostat & galvanostat devices)

a) For creation of non homogeneous electric field (and field gradient) the different kinds of wedges (hyperbolic, linear) are proposed.

Hyperbola is described by formula y=a/x . Hyperwedges is cut from gel with coefficient of a. The choice of wedge's type depended on separated macromolecules mixture (there are no universal wedges size).

Range for V1- (-500V, 0V)

Range for V2 - (0V, 500V)

Range for T1&T2: (1sec, 1000sec)

Inverse Focusing ("START" Method)

As provided herein, this method provides for improved focusing and resolution of the separated molecules. The method comprises a particular case of conductivity dependent, non-uniform field electrophoresis and is based on the generation of a steep field jump by proper selection of the electrical properties of two kinds of gels with different conductivity. Application of an electric voltage as in regular electrophoresis to a bi-layer of two gels will produce a field jump (see Fig. 1 & 25). The field jump when positioned at the start position of the electrophoresis cell will cause a compression of the fractions in the analyzed solution. The subsequent electrophoresis process will separate the fractions with improved resolution.

Geometrical Traps:

In addition, the present invention provides a method of separation of macromolecules in a media using non-uniform electric fields based on the design of geometrical features of the electrophoretic media. The specific details of the design of geometric traps consist of choosing the geometry of the electrophoretic cell. Let us consider the one-dimensional geometry of a cell, where crossection of the cell S is changed along the x coordinate S=S(x). Becuase of total current J is constant in all crossections, and it is proportional to ES(x) (J~ES(x) = const),we obtain that electrical field is changing along the cell:

$$E \sim S^{-1}(x) \qquad (1)$$

Let us consider the drift velocity of a charged particle under the applied field strength E(x):

$$U_d = bE(x) = U_d(x) \qquad (2)$$

Where b is the mobility of the particle, and is slowly dependent nonlinearly for electric field strength E in common case. As a result, the drift velocity for different fractions must be more different then in regular electrophoresis process. Changing the geometry of the cell we shall obtain more separation ability of the regular electrophoresis.

Fig. 2 corresponds to hyperbolically changing of cross section area $S(x) \sim x^{-1}$, which results in linear dependence for the electrical field strength E~const-x.

Fig. 3 shows an electropherogram of fragments of hydrolyzed DNA lambda phage HIND III in Hyperwedge 0.6% agarose gel. Voltage 20 V.

In the hyperbolic field all small fractions of macromolecules stop at the wide end of the vessel due to the fall of the field strength. This effect we call as „the geometric trap". The shape of the cell is calculated by accounting for the properties of the gel and using methods of conformal mapping for optimal shape based on the expression (1) and (2). For standard separation processes a set of vessels can be designed and manufactured. Another embodiment is a set of basic shapes and construct the optimal configuration by a modular like assembly. The schematic result of geometric fraction trapping is show on Fig. 16 and 20.

Fig. 17 presents schematic results of electrophoretic separation of the fractions of macromolecules. The result of application of the geometric traps is more uniform in the scale of fractionation. This feature is very useful for the case of high weight differences for sample fractions.

Hyperwedge Focusing:

In the hyperwedge the electric potential V(x,y) and strength field E is defined by potential gradient that gives the following expression (Fig. 2):

$$V(x,y) = -V(t)(x^2-y^2)/L^2 \qquad (1)$$

$$Ex=2xV(t)/L^2, \qquad Ey=2yV(t)/L^2 \qquad (2)$$

Where V(t) is the effective voltage on the gel and L is the length of the hyperwedge segment. The x component of the electric field is changed linearly along the wedge. The availability of the y-component of field tends to the fractions stretching across the wedge's symmetry axis but in the vicinity of symmetry axis (y=0), y-component is small. It is interesting to note that Ex does not depend on coordinate y, what must put in the straight line form of fraction fronts with electrophoresis in hyperwedge.

$$\frac{dx}{dt} = < U_d >$$

$$x(t) = \sqrt{\frac{\alpha}{C\exp(-2\alpha t) + \beta}}$$

$$x_f = \sqrt{\frac{\alpha}{\beta}} = L^2 \sqrt{\frac{-b < V >}{4\mu < V^3 >}}$$

The focusing properties of the hyperwedge are symmetric relative to the symmetry axis therefor one hyperwedge can be used for two independent separation procedures.

Impulse-Field (meander):

Reference is now made to Fig. 12, during the field E_1 action the macromolecule drifts along the axis X and under the action of the weak field E_2 operating for a more extended time T2 it is shifted to a reversible direction.

In the present invention, the gel tank is preferably of conventional design comprising an open-topped rectangular box made of an electrically insulating material such as glass, Plexiglass or Perspex. Said gel tank is filled with an electrophoresis buffer solution. Preferably the electrophoresis buffer solution is a conventional electrophoresis buffer solution. Advantageously, said gel tank is provided with means for circulating, and thermostatically controlling the

temperature of, said buffer solution. Said retained gel may consist of any electrophoretic gel capable of allowing transport of large molecules. Typically said retained gel is an agarose gel. Agarose gels are typically cast between glass plates with sample wells formed by insertion of a well former in the cassette before said agarose gel solidifies. Samples of molecules for electrophoresis may be loaded onto said agarose gel in agarose blocks before immersing said agarose gel in said buffer solution. Alternatively, said samples may be loaded onto said agarose gel as solutions after immersing said agarose gel in said buffer solution. Said gel retaining means may comprise any conventional means for retaining and supporting said retained gel in said gel tank. US. Patent No. 4,737,252, U.S. Patent No. 4,473,452, U.S. Patent No. 5,167,784, U.S. Patent No. 5,495,519, U.S. Patent No. 5,135,628, Monthony et al. U.S. Pat. Nos. 3,948,743; Delony et al. U.S. Pat. No. 4,574,040; Cantor et al. U.S. Pat. No. 4,861,448; Hochstrasser U.S. Pat. No. 4,874, 490; Kushner et al. U.S. Pat. No. 4,954,236; Fernwood et al. U.S. Pat. No. 4,994,166; Chu et al. U.S. Pat. No. 5,073,246, and US Patent No. 5, 167,790 and US Patent No. US5453162, are hereby incorporated by reference herein.

In one embodiment the gel is retained in the form of a wedge geometry having the following dimensions13 cm in length, 10 cm in width and 0-4.4 cm in height. The hyperbolic wedge shape is shown in Fig. 2.

In general a wide variety of sizes and dimensions with varying narrow/wide ratio wedges have been employed with wedge lengths from 100 microns up to 500 millimeters.

Optionally, said retained gel may be additionally secured and supported by removable glass bars adjacent the respective vertical edges of said retained gel. If said retained gel is an agarose gel it must be retained in position against the natural buoyancy of said agarose gel in said buffer solution. Said means for supplying the a non-uniform and time dependent (periodical) electric field between said cathode and said anode may consist of a conventional power source, typically with a maximum output between 300 mA, 150 V and 2.5 A, 500 V. Said means for supplying an alternating polarity potential difference to

said electrodes may consist of a conventional power source, typically with a maximum output between 300 mA, 150 V and 2.5 A, 500 V, in conjunction with a switching unit capable of alternating the polarity of the potential difference, supplied by said power source, with a pulse time of between 0.1 seconds and at least 5000 seconds. Preferably, the switching unit is capable of alternating the polarity of the potential difference with a pulse time of between 60 seconds and 60 minutes. Optionally, both said power sources may be the same power source . Preferably, said means for supplying an alternating polarity potential difference comprises a power source and an electronic switching unit capable of supplying and ramping an alternating polarity potential difference with a pulse time of between 0.1 seconds and at least 5000 seconds, more preferably, between 60 seconds and 60 minutes. In preferred embodiment the pulse time is 5 to 15 seconds and 20-90 V.

The material of construction of said anode, cathode and electrodes may be any suitable electrically conducting material, such as platinum or graphite. Preferably said anode, cathode and electrodes are platinum.

As presented in Fig. 14, there is shown a schematic drawing of a standard electrophoresis apparatus 10 having a power supply 12, an electrophoresis gel system including the tank 85 and a means 42 connected together for controlling the electric field force, electric field angle and the pulse duration to resolve DNA molecules greater than 1,000 kb in length along straight, unbent lanes within a gel. The electrophoresis system 10 permits adjustment of the field period and voltage. The electrophoresis gel system includes the shallow electrophoresis tank 85 which is made out of insulating material adapted to contain electrolyte buffer 86. Completely submerged in this buffer is a square sheet of agarose gel 90 containing a number of wells or oval depressions 91. In these wells are plugs of gel containing mixed DNA to be separated. To create the field in the gel separating system, electrodes 71 through 82 provide electrical contact to the buffer from the power supply 12 through the switching means 42. The electrodes are preferably made of an inert metal such as platinum.

The electrophoresis system of Fig. 15, is illustrated by a series of interconnected components comprising an electrophoresis chamber or gel box 10, a pump 11, a heat exchanger 12, a switching means 13, a DC regulated power supply 14 and a timing device 15. In the schematic diagram of FIG. 15, a top view of the gel box is illustrated in which the gel layer or slab is hyperbolic in geometry 16. The longer arrow and larger polarity signs (+ and -) indicate the predominate condition. That is, in variations in which net migration is achieved by applying the same voltage in both directions, the predominant condition is one that is applied for the larger fraction of each switching cycle; in variations in which different voltages are applied for the same interval, the predominant condition would be the higher voltage. The usual convention of arrows pointing from + to - signifying the electrical-field (E) is employed in the figures. Because most macromolecules, including DNA, are negatively charged under electrophoretic in the direction of migration is in the opposite direction of the large arrows. The gel box comprises a generally rectangular sided chamber having sidewall 20, endwalls 22 and 23, and base portions 24 and 25. A front sidewall which would lie opposite the rear sidewall 20. The gel box is further provided with a raised platform or tray 28 in a plane below the top of the gel box and supported at opposite ends by partition walls 26 and 27. This platform serves as a support for the gel layer 16. The side-, end-, and partition-walls at each end of the gel box also form buffer chambers in an amount sufficient to cover the gel layer as shown by the buffer level 32. Electrodes 33 and 34 made of electrochemically inert material and having suitable electrical conducting properties, for example platinum, are provided for retention within the buffer chambers 30 and 31 respectively. They are preferably positioned along the endwalls at the bottom of the buffer chambers with electrical leads 35 and 36 for connection to the switching means 13. Tubing 37 and 38 with openings into buffer chambers 30 and 31, respectively, are provided for re-circulation of buffer from the gel box through a heat exchanger 12 by pump 11. The heat exchanger serves to dissipate heat generated within the gel box during electrophoresis. The cooling fluid source 39 for the heat exchanger can be provided by a conventional re-circulating,

refrigerated water bath. The switching means 13 is critical to the provision of the periodic field-inversion of the gel electrophoresis. This system in essence can comprise power relay device. FIG. 23 is a circuit schematic that indicates the manner in which the power relay can be wired.

The power supply can be any suitable source of direct current. The apparatus is in a configuration that allows non-uniform and time dependent (periodical) electric field to be carried out at a constant applied voltage with a larger portion of the switching cycle devoted to forward migration than to reverse migration. In variations in which a higher voltage is applied in one direction than the other, more complex electrical circuitry is required. For example, two power supplies can be employed, wired through separate power relays to independently programmable output circuits of the timing device.

Various components which can be used in the gel electrophoresis apparatus of this invention are commercially available. For example, gel electrophoresis chambers for use in the horizontal mode can be obtained from various sources such as Bethesda Research Laboratories (Gaithersburg, Md.) Model 144 Horizontal Gel System; Bio-Rad (Richmond, Calif.) Model 1405 and 1415 Electrophoresis Cells; Pharmacia (Uppsala, Sweden) FBE 3000 and GNA-200 Flatbed Cells; and the LKB (Bromma, Sweden) 2117 Multiphore II Electrophoresis Unit. Such devices can be adapted for use in the invention by appropriate combination with the other components specified herein to provide the periodic field-inversion.

Alternatively, the electrophoresis box can be readily fabricated from rigid materials such as, for example, acrylic plastic. Thus, a conventional laboratory scale gel box can be constructed from 0.25 inch thick clear acrylic plastic with inside dimensions 8.5.times.14 inches as viewed from the top. The gel platform can be 8.5.times.8.5 inches set in a plane 1.5 inches below the top of the gel box. Buffer chambers at the two ends can extend to a depth of 3. 4 inches from the top of the gel box. Electrodes 8.5 inches log, 100% platinum (26 gauge), can be set directly against the intersection of the end walls and the bottom of the buffer chambers.

For a gel box of the foregoing size, buffer can be suitably re- circulated at a rate of about 250 ml/minute using, for example, a Cole Parmer (Chicago, Ill.) Masterflex T-7553-00 drive with a T-7018-21 head equipped with silicone tubing with 5/16 inch inner diameter.

The invention provides separation and resolution of nucleic acids such as DNA, RNA, cDNA of various base pairs and genomic DNA and chromosomes. For example, DNA sequences up to approximately 500-20000 bps; and DNA in the 100,000-10 million bp range ("chromosome mapping") are demonstrated. Furthermore, this invention provides separation and resolution of intact chromosomes ("chromosome sorting"); and resolving DNA conformers (DNA molecules of the same size, but different composition or shapes). This could be very valuable for scanning mutational analysis or to map out single nucleotide polymorphisms ("SNPs"). Increase throughput (5-10 fold increase in speed without concomitant loss of resolution) is also very valuable.

Separation of large DNA fragments

Existing methods of separation of large fragments of DNA (>100kb) based on FIGE and CHEF are slow and tedious. It is the subject of this invention to bring an improved method of

separation of large fragments which shortens the separation time considerably from many tens of hours to several hours.

In this embodiment of the invention a dramatically improved method of separation of large DNA fragments (>100kb) is achieved by applying time varying electric potential in a variable cross section separation gel (hyperbolic wedge).

This new method is based on improved understanding of the motion of large DNA fragments in gel, the mutual interaction between gel and DNA fragment and forces acting on a DNA fragment in a time varying electric field. Specifically the understanding of frequency dependence of the net drift velocity of the DNA fragment (far from the resonance frequency) makes possible the identification of frequency regions for which the net drift velocity of specific

fragments is high. An example of a possible approach which can explain the motion of large DNA fragments in a gel was proposed by J. M. Deutsch, Science Vol. 24, 1988 p. 922.

Fig. 18 and 23 presents the result of separation of large DNA fragments by the method of invention together with a comparison with standard method of separation by PFGE. It is clearly evident that the invention provides for much shorter separation times.

Another example of fast separation by the method of invention is presented in Fig. 20.

The existence of non constant field and of variable media conductivity results in uneven heating of the gel that requires additional ways to remove the heat. Besides care need to be taken to prevent diffusion blurring due to concentration gradient of electrolyte in gel. For that purpose the gradient region should be insulated with a glass plate from the covering buffer solution. The standard photo plates was used in our experiments. Despite this complication a great improvement in separation can be achieved by a relatively minor change relative to the conditions of standard electrophoretic separation. This is manifested particularly for solutions in which small and large DNA fragments have to be separated and identified.

Two buffers are commonly employed for PFGE--TAE and TBE (1x TAE is 40 mM Tris acetate, 1 mM EDTA, pH 8.0; 1x TBE is 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Both are used at a relatively low ionic strength to prevent heating and carry the designations of either 0.25 and 0.5x to indicate the dilution relative to the standard concentration. An added benefit to low ionic strength buffers is an increase in DNA mobility. For example, while using RGE to compare various buffers and agaroses, White (1992) found that lowering both TAE and TBE to 0.25 x gave the maximum mobility (40-50% faster than 1x). Below 0.25x, the DNA mobility dropped off.

The type of agarose also affects DNA separation, with the fastest mobilities and best resolution achieved in gels made of low electroendosmosis (EEO) agarose (Birren, et al., 1989; and White, 1992). Although most standard

electrophoresis grades of agarose are suitable for PFGE (e.g., SeaKem GTG), agarose with minimal EEO will provide a faster separation. Several low EEO "pulsed field grades" are available, including FastLane and Gold (FMC BioProducts), and Megarose (Clontech).

As is the case with conventional electrophoresis, in field- inversion gel electrophoresis, large numbers of samples that have been loaded onto adjacent lanes of a single gel will migrate in parallel with one another, experiencing closely comparable electrophoretic conditions. The ability to make reliable, lane-to-lane comparisons between many samples on the same gel is one of the strongest features of conventional electrophoresis.

The applications of the field-inversion technique are not limited to DNA. The qualitative electrophoretic behaviour of other charged macromolecules such as RNA, protein, nucleoprotein particles, and protein-detergent complexes is generally similar to that of DNA.

Generally, the sample is run in a support matrix such as paper, cellulose acetate, starch gel, agarose, or polyacrylamide gel. Such support matrixes are known to those skilled in the art. Agarose is a highly purified polysaccharide derived from agar. Unlike agar, it is not heavily contaminated with charged material. Most preparations, however, do contain some anions such as pyruvate and sulfate, which may cause some electro-osmosis. Polyacrylamide is chemically complex, as is the production and use of the gel. Polymerization of a polyacrylamide gel is accomplished either by a chemical or a photochemical method. In the most common chemical method, ammonium persulfate and the quaternary amine,N,N,N',N' tetramethylethylenediamine or TEMED, are used as the initiator and the catalyst, respectively. In photochemical polymerization, riboflavin and TEMED are used Shining long wavelength ultraviolet light, usually from a fluorescent lamp, on the gel mixture starts the photochemical reaction. Since only a minute amount of riboflavin is required, photochemical polymerization is used when a low ionic strength is to be maintained in the gel.

The present invention can be conducted using power supplies, electrodes, gel media, electrophoresis chambers, and other elements as found in known devices, combined as taught herein. One preferred embodiment of the power supply means comprises two separate power supply units connected to a high voltage switching unit which is connected to electrodes A and B, while a third power supply is connected to electrodes B and C. A more preferred embodiment comprises two commercial programmable bipolar operational power supplies (BOPS). One BOPS is connected to electrode pairs A and B, the other to electrode pairs B and C. The system is controlled using a computer. A preferred arrangement comprises a digital computer equipped with an IEEE-488 Board and an IEEE-488 to analogue converter (Kepco SN 488-122), which controls a Kepco Bipolar Operational Power Supply (BOP) (Kepco BOP 500-M)(Kepco Inc. , Flushing, N.Y.). The power relay can be, for example, a Deltrol Controls (Milwaukee, Wis.), Series 900 DPDT No. 20241-83. For higher voltages or faster switching intervals, various other switching devices are available such as vacuum relays, solid-state relays, and the like. Illustrative power supplies are the Heathkit (Benton Harbor, Mich.) 18-2717 Regulated High Voltage Power Supply and the Hewlett Packard (Berkeley Heights, N.J.) SCR-1P Model 6448B DC Power Supply.

The present invention also provides an apparatus for separating larger particles from smaller particles, which includes a container for holding a medium in which, the particles are suspended. The container has an inlet and an outlet and is disposed between two opposing primary electrodes. The two opposing primary electrodes are connected to a primary switching unit. The switching unit is connected to a controller. The two opposing primary electrodes generate a non-uniform electric field across the medium in a forward direction from the inlet of the container towards the outlet of the container. The primary direction defines the direction in which the particles travel through the medium. The two opposing primary electrodes also generate a non-uniform electric field across the medium from the outlet towards the inlet. The apparatus further comprises at least one pair of secondary opposing field generators disposed on opposing sides of the

container at a secondary angle with respect to the inlet and the outlet. The secondary field generators are connected to a secondary switching unit. The controller sends signals to the primary and secondary switching units to apply the electric field, thereby creating a field environment.

In an embodiment, the apparatus comprises two pairs of secondary opposing field generators. The electric flow is generated by the power supply 1. Any voltage the system can tolerate may be used, e.g. 100 to 10000 volt, especially 500 to 10000 volt, preferably 500 to 5000 volt, e.g. 500, 1000, 5000 or even 10000 volt, provided the generated heat can be dissipated by proper cooling. At equilibrium, typical values are e.g. 1000 volt, 3 mA and 3 W or 500 volt, 10 mA and 5 W.

The electrophoretic matrix is a carrier for the electrophoretic separation. The hydraulic flow is generated e.g. by a pump, by stirring or by rotating the flow chamber 8 around a suitable axis and comprises as liquid phase a solution containing the mixture to be separated.

A qualitative molecular interpretation of these results can be made on the basis of measurements of the instantaneous velocity of linear DNA after field inversion (Platt and Holzwarth, Phys. Rev. A, 40, 7292, 1989), video micrographs of DNA during gel electrophoresis (Smith et al., Science 243, 203, 1989), and computer simulations of the motions of DNA during electrophoresis in gels (Deutsch and Madden J. Chem. Phys., 90, 2478, 1991; Zimm, J. Chem. Phys. 94, 2187 1991).

After the electrophoresis run is over, the gel is usually analyzed by one or more of the following procedures which are known to those skilled in the art: staining or autoradiography followed by densitometry; or blotting to a membrane, either by capillarity or by electrophoresis, for nucleic acid hybridization, autoradiography or immunodetection. The most common analytical procedure is staining. Protein gels are most frequently stained with Coomassie Blue or by photographic amplification systems using or other first row transition metals. Coomassie Blue staining is only sensitive to about 1 µg of protein; whereas, the photographic amplification systems are sensitive to

about 10 ng of protein. Once the gel is stained it can be photographed or scanned by densitometry for a record of the position and intensity of each band Nucleic acids are usually stained with ethidium bromide, a fluorescent dye, which glows orange when bound to nucleic acids and excited by UV light. About l0-50 ng of DNA can be detected with ethidium bromide. These gels are usually photographed for a record of the run.

A second common analytical procedure is autoradiography. It is used to detect radioactive samples separated on a slab gel. This procedure requires that the gel be first dried to a sheet of paper and then placed in contact with x-ray film. The film will be exposed only where there are radioactive bands or spots. The resulting autoradiogram is usually photographed or scanned by densitometry. The highly sensitive technique, blotting, is used to transfer proteins or nucleic acids from a slab gel to a membrane such as nitrocellulose, nylon, DEAE, or CM paper. The transfer of the sample can be done by capillary or Southern blotting for nucleic acids (Southern, 1975) or by electrophoresis for proteins or nucleic acids. Southern blotting draws the buffer and sample, usually DNA, out of an agarose gel by placing the slab in contact with blotter paper. A nitrocellulose or nylon membrane, layered between the gel and the blotter paper, binds the nucleic acids as they flow out of the gel. And since the membrane binds the DNA or RNA in the same pattern as on the original gel, the result is a faithful copy of the original. But Southern blots usually take a long time, frequently 10- 20 hours to prepare.

Lab on chip

As provided herein, this invention provides as a variant of Lab-Chip techniques the special types of electrophoretic capilllary shapes (For example, United States Patent 5,582,705, issued December 10, 1996 entitled „Multiplexed capillary electrophoresis system" incorporated by reference herein) have been designed for focusing. The adaptation of focusing method for the capillary electrophoresis requires a capillary with varying cross section along the capillary. The capillary sections are calculated similarly to the wedge. The traditional methods of electrophoresis are performed in one or two dimensional

gel media. The "dimensionality" of separation system may be extended to - dimensional coordinate structures formed in the plane and enabling multi step separation and handling. Methods of construction such a chip are known to those skilled in the art. The advantage of this method in comparison with the "Lab on Chip" method is that instead of using pre designed single use patterns an optimal pattern can be generated on demand.

For creating the large quantity of barriers which non homogeneity of electric field (gradient of field) takes place the following gel geometry (dimension) is proposed. It is proposed to do „labyrinth" from gel. The „labyrinth" is gel segments which are cut out at a right angle (at an angle of 90 degrees). The electrodes are applied to the outset and to the end of „labyrinth". The field gradient appears in the positions in which the gel turns through 10- 90 degrees. The field gradient is necessary to nonlinear mobility separation.

A particular method to produce multiple channels is based on the application of non polymerized gel to the surface with a special "pencil". The "pencil" consists of a volume filled up with non polymerized gel. The "pencil" is a part of a plotter. The plotter control is performed with PC. To operate the plotter the special software is used. Before hand applied gel is cut out according to the special software. The special regions determined by researcher are cut out on the prepared surface. The "knife" operation is performed with special software.

Apparatus and Method for Fractionation, Separation and Focusing of cells and cell fragments

A further embodiment of the invention is represented by an apparatus and method for fractionation, separation and focusing of cells.

This embodiment of the invention provides for a novel method and apparatus for separation, purification, manipulation and focusing of large bio particles and bio macromolecules, such as chromosomes, cells, large DNA and RNA fragments and other components of cells. The method is suitable for the separation of particulate matter in the size range between 1 nm up to 1 mm both for bioparticles and for non-biological, atomic or molecular assemblies placed in liquid, conductive media.

The need for cell separation arises in many areas of medical diagnostics and treatment and biotechnology. Examples of such applications are separation of malignant cells from healthy cells, separation of fetal cells from maternal blood samples, isolation of mutant cells during strain development and others.

The general formalism applied to the new method of cell separation is based principally on dipolar forces caused by varying electric fields.

In general a dipolar force acting on a dielectric particle is given by:

$$F_d = (p\nabla)E$$

where p is the dipole moment and E the nonhomogenous electric field.

The effective dipole moment of a particle consist of the permanent dipole p moment and a induced dipole moment and is given by:

$$p = p_0^2 E/3kT$$

k being the Boltzman factor and T the temperature.

The forces acting on a charged particle in a nonhomogenous electric field will consist of the following

$F_q = qE$ (Coulomb force)

$F_d = (p\nabla)E$ (dipolar force)

And $F_f = -6\pi a\eta U_d$ (friction force in a viscous medium, U_d being the drift velocity, a the diameter of the particle and η the viscosity coefficient of the medium)

The general expression for the drift velocity of the particle is given by:

$$U_d = [qE+(p\nabla)E]/6\pi a\eta$$

For field values applied in normal electrophoretical separation and for charge values characteristic for cells the coulomb term of the force is much larger generally than th dipolar force which makes electrophoretic separation of cells not efficient.

It is the subject of this invention to utilize time varying nonhomogeneous electric fields to enhance the dipolar term.

For example if we use a field with time average $<E>=0$ only the dipolar tem will cause the movement of particles and allow separation.

In this case :

$$U_d = (p_0^2/3kT)\nabla E^2/6\pi a\eta$$

It is this expression when utilized in the appropriate conditions as described below which allows for efficient separation and sorting of particles like cells and other biomolecules.

The particular embodiment of the present invention is described in greater detail herein below.

The invention consists of a novel realization of a combined electrophoresis and dielectrophoresis system for the separation of quasi neutral or neutral particles and specifically cells. The separation is achieved by means of applying non homogenous electric fields generated by applying a non uniform electric potential across a conductive medium (buffer solution, electrolyte) and by introducing spatial field non homogeneity by utilizing separation vessels with nonrectangular shape and in general possessing a variable cross section.

The system of the invention consists essentially of the following:

1. A power supply with a control system
2. A specially shaped separation cell for separation and extraction of cell fractions
3. A detection system based on an optical microscope.

Sorting of cells (or other particles) is achieved by means of application of nonhomegenous electric fields to a sample of cells placed in a conductive liquid.

The general diagram of the separation system subject of this invention is presented in Fig. 26

The system consists of a separation chamber filled with a conductive liquid (electrolyte, buffer solution) suitable for the separation of cells, a controllable power supply with a wide dynamic range of voltage, current and power, methodology for the generation of the non homogenous electric fields (computer controlled or manual) and a cell fraction detection and recovery system.

In one embodiment of this invention the separation is shaped as shown in Fig. 27. The separation chamber consists of a narrow channel with a variable cross section, its boundaries being for example shaped as a hyperbola and depth of 1-2 mm. The width of separation channel is limited to several millimeters to prevent or minimize convection currents. For example the width of hyperbolic channel of 32 decreases from 5 mm at one end down to 2 mm on the other end, the total length being for example 5 cm. Two metallic electrodes are placed in a wide section of the chamber at both ends.

In another embodiment of this invention the separation chamber is shaped as a miniature symmetrical double triangular wedge with a narrow opening in the center as shown in Fig. 29 The dimensions of the opening are about 50 microns . By applying a variable square shaped potential to electrodes placed at the wide ends of the separation vessel large field gradients are generated in the narrow region allowing for separation of cells with different dialectric properties as shown in example.

It is clear that separation chambers with a variety of shapes and channel cross sections can be designed to achieve desired non-homogenous electric fields. These channels can have cross section shape like for example rectangular, circular like a capillary or any regular and non regular shape. Generally the variable cross section vessels or separation chambers or channels are designed in shapes and dimensions to fulfill the requirement for specific field inhomogenuity with a programmed linear dependence along the vessel. Some

additional examples of variable cross section separation chambers are shown in Fig. 30(a-o).

A very powerful realization of this invention are the miniaturized channels which basically form a microfluidic system which can have a multiplicity of channels with identical or varying dimensions to allow for manipulation ,sorting and detection of cells.

For the purpose of detection an optical microscope is focused at a certain point in the separation chamber for visual detection of moving cell fractions.

In another embodiment of this invention specific particles, for example cells can be reacted with fluorescent ligands (stained) as known in the art and detected by illuminating the channel with ultraviolet light.

In another embodiment the detection system can consist of an illumination system for example a laser and the separated particle fractions are detected by measuring the scattered light intensity as known in the art.

As in the case of DNA separation the use of combined electrophoretic and dielectrophoretic forces enable the creation of virtual traps. These traps appear at points along the channel where the total velocity due to electrophoretic and dielectrophoretic forces acting on a specific fraction is equal zero.

In such a way this invention makes possible the accumulation and focussing of specific fractions in preset locations along the separation channel for fast detection and extraction. These locations of focusing traps can be changed by manipulation of the electric voltage parameters.

As in the previous embodiments detection and visualization methods known in the art are applied here.

In another embodiment of this invention the separation chamber is designed to enable fast extraction of separated fractions for further analysis and diagnostics. In this design along the separation channel one or several perpendicular separation channels are added Fig. 31 By applying voltage forms

across these perpendicular channels fractions passing through the main channel or focussed at these channel position can be extracted.

Traveling Concentration Waves (TCW) for large particle separation

This specific embodiment of the present invention provides for a new dielectrophoretic separation technique for improved separation and manipulation of bioparticles in an extended range of sizes and shapes. More specifically particles can be separated according to their mass, density, internal and surface charge distribution, shape and dielectric properties.

Sorting of molecules is performed by the means of Traveling Concentration Waves (TCW). Several types of TCW can be realized, the main example being pH waves or the moving isoelectric points (MIP). Fig. 32 presents a schematic shape of such a wave generated by applying a potential waveform presented in Fig. 35 in a separation cell presented in Fig. 38.

In this method, the nonionic analytes are separated into sharp zones in a separation chamber of a variety of shapes for example an elongated (cuvette) filled with a gel and buffer system enclosed between two electrodes. Another example is a rectangular chamber like in regular electrophoresis or a tube like segment enclosed between two or more electrodes. Other shapes are possible as well and as such the invention is not limited to these particular shapes. These chambers can be constructed of glass, polymeric materials, plastics and other materials as well. For achieving separation the electrodes are energized by being subjected to an alternating potential form of special design. This alternating potential creates a periodically moving region of a high electric field gradient acting on the sample .The separation occurs according to the nonlinear electrophoretical mobility of the separated molecules.

In one variation of the embodiment of the present invention a single buffer solution for example TAE (Tris-Acetate EDTA) is subjected to a variable potential which generates alternatively at each of the electrodes regions rich in

H^+ ions and regions rich in OH^- ions. These regions originate at the electrode and expand along the vessel.

Since the electrophoretic mobility of the H^+ is very large the expansion is very rapid and the leading edge very steep. This wave front when reaching the analyte sample injected in the vicinity will act on the sample and cause the separation of the sample according to its molecular constituents and their dielectric properties. On changing the polarity an OH^- wave starts expanding and neutralizes the hydrogen ions. Since the mobility of the OH^- ions is much lower (~80x lower) the wave front will be very shallow, the drift time much longer and the force acting on the sample will be negligible.

By repeating this process many times an efficient separation procedure is established resulting in a very fast separation of large bioparticles.

A further variation of this embodiment of the invention enables the separation of proteins by their isoelectric points. By selecting a buffer solution with a predetermined pH and by controlling the current one can design a pH step which will immobilize all proteins with IP in the pH interval while all other proteins will be separated by the wave front.

This mode is particularly suitable for particles like proteins in which the main dielectric interaction is with the surface.

Another variation of this embodiment of the invention is realized by using two immiscible or slowly interdiffusing buffer solutions with different cation ions: one with H+ and the other with for example K^+. By filling a cuvette with equal amounts of the buffer solutions a sharp boarder zone is created in the center of the vessel The application of an electric potential to the electrodes inserted in the buffer solutions at both ends will create a sharp electric field jump at the interface between the buffers. If the buffer solutions are selected in such a way that the fast cations when crossing the interface are abruptly slowed down the effect of applied electric field will cause the appearance of a charge wave crossing the interface. This effect is similar to a shock wave generated when very fast moving particles expand into a slowing down medium.

This charge wave will generate a very high local field and field inhomogenouity which will move under the applied potential. Biomolecules placed on the way of this moving charge will be subjected to the force generated by the electric field.

Reversing of the electric potential will result in a fast decay of the charge wave.

Alternating the potential will cause efficient separation of the fractions in the analyzed sample.

Separation of proteins

In this embodiment of the invention shaped separation vessels were employed for an improved separation of proteins.

Separation of proteins by their net charge can be attained by several electrophoretic systems.

However most of the problems associated with existing systems are related to the stability of the electric field and thus the stability of proteins running in this field for rectangular shaped vessels.

It is the subject of this invention the achievement of stabilized electric fields by the use of hyperbolically shaped boundaries of the separation vessel.

By using these vessels more distinct protein bands are obtained and more proteins can be visualized. The shaped geometry enhances protein migration in different buffer systems including continuous, moving boundary and isoelectric focusing techniques.

For example monoclonal antibody was separated by discontinuous and moving boundary electrophoresis on both regular and hyperbolically shaped gel geometries. Fig. 37

In the moving boundary method the antibody the antibody runs faster in the shaped geland only in this gel there is a good separation of the protein into three distinct bands (Fig. 38 left panel).

In the discontinuous system the protein migrated a similar distance, however, separation was better in the wedge shaped gel and three antibody species could be detected.

These bands were also visualized in isoelectric focusing and confirmed the separation result.

In another example moving boundary electrophoresis was used to separate human plasma.

A comparison between rectangular and wedge shaped geometry under similar electrophoresis conditions shows that the protein bands in the shaped wedge are more distinct and more proteins can be visualized. Furthermore less streaking of proteins is observed Fig. 39

Examples for DNA separation

The simplicity of the method of invention, high resolving power and ease of combining with other techniques makes it a very powerful and potential technique for wide application. The invention comprises the method, the design and device for nonlinear electrophoresis based on several methods of obtaining special electric field patterns for separation and manipulation of macromolecules. The methods comprise: 1. The generation of "virtual traps" for DNA focusing by producing special electric signals generated by a computer driven voltage generator 3. The generation of steep field jumps by gel composition for inverse focusing.4. Combination of several of the above methods or the creation of a "Lab on Chip" concept for manipulation of biomolecules and particles.

This invention relates to an apparatus and methods, by which a separating procedure and system can be produced for the manipulation of selected molecular species. The method is based on the formation of a two dimensional, multi segment trajectory constructed from separating media in which each segment is designed to perform a certain separation procedure. In such a way a multi step separation is performed resulting in the trapping of selected molecules at a predetermined site.

Materials and Methods:

Tris-Acetate or tris-phospate buffer solution (0.04 tris-acetate or tris-phospate + 0.002 EDTA) is used as the working buffer. To stain the DNA fragments in the UV range Ethidium Bromide or Crystal Violet (Sigma USA) are applied. The agarose gels prepared for the above experiments were based on the agarose for DNA electrophoresis produced by BioRad Lab. Electro-endo-osmosis parameter Fritsch and J.Sambrook Laboratory Manual "Molecular Cloning" (Cold Spring Harbor Lab 19 used in these measurements was m= - 0.1. The experiments were conducted according to T.Maniatis, E.E. 82)

Example 1

Fraction's compression in linear wedge at constant Potential

The surface for gel deposition is placed at an angle to horizontal which causes the formation of a the wedge when the surface is being filled by agarose gel (0.6%). One end has a thickness of 0.2cm, the other end is of 1.7cm. The wedge is covered with buffer solution of 1XTAE (tris- acetate and EDTA) with a layer of 0.2 cm thick , 5.5cm long and 4.0cm wide.

Effective thickness of the gel ends are of 1.9 and 0.4 cm. The marker DNA lambda-phage HIND III is injected into the special start pockets, which are located at the distance of 6.5 cm. from the thick end of gel. Then a voltage of 10V is applied to the electrodes. The three fractions of DNA go through the distance of 1.4 cm, 2.2 cm, 2.6 cm respectively. Since the electric field falls monotonically in the direction of the wide gel end focusing of the various fractions is observed (Fig. 24)

Example 2:

Fraction's compression in a hyperbolic wedge at constant Potential

Two hyperbolic inserts from polymethyl acrilate are placed in the cell with the following characteristics: the length is 13 cm, the width is 10 cm. The insert dimensions vary according to a hyperbolic with parameters such that the hyperbola narrows from 0 at one end to 4.4 cm at the other end. Agarose gel (0.6%) is poured into the volume between the inserts, creating a gel shaped as a hyperbolic wedge ("hyperwedge") with the length of 8.4 cm and the width of from 10 cm in the widest part to 1.2 cm in its narrow part. The wedge is covered by buffer solution of TAE (height 0.2cm). The marker DNA lambda-phage is introduced into the start pockets, located at distance of 1.5 cm from the narrow edge. When a constant potential is applied alinearly decreasing field from the narrow to wide end of the hyperbolic wedge provides for the focusing of DNA fraction (see Fig. 25).

Example 3:

Nonlinear focusing in the hyperbolic wedge with time varying potential (MEANDER)

The hyperwedge, which is described in the Example 2, is used for nonlinear DNA focusing. In this Case a variable in time. periodic signal of the special type (meander) is applied to the electrodes. The average for period electric field is relatively little (less than 2V/cm), in comparison with field amplitude during the period of the meander action (less than 20V/sm). The sample DNA is entered into the start pockets which are placed in the "narrow" end of agarose gel (0.6%). At the process of nonlinear focusing the more heavy fractions move to the direction of the wide gel end, while the light ones remain behind. With the different parameters of themeander voltage they can begin to move to the opposite direction from the start. The behavior differ sharply from all the traditional methods of electrophoretic DNA separation where everything is determined by ratio charge/size. When the observation times are more than 24 hours, the focusing in the so-called virtual traps is can be seen at position where the fraction velocity goes to zero (Figs. 8, 9, 10, 11, 12).

Examples of cell separation

Example 4.

Consider an electrophoretical cell, partitionned across non-conductive dielectric wall. There is a hole with the diameter 50-μm in the wall. The wall divides the cell in to two cameras: right and left one . There is an electrode in each camera, to which variable in time voltage is applied with zero average voltage. Both cameras near the holes filled with the electrolyte (buffer).A sample, containing a mixture of RBC, is placed into the camera. During presenting a periodic voltage with zero average on electrodes of cell, in the field of holes will be observed greater gradients of squared electrical field. As an effect, on RBC acts a dipole power, forcing them to drift in the area of strong field. This phenomena is demonstrated in Fig. 40.

RBC concentrate near the "hole", when a variable signal. is applied. Symmetrical and asymmetrical signals were used (Fig. 12 a, b). Type of the "hole" is depicted in Fig. 30 h.

Notice that in particles, if induced dipole moment exceeds own dipole moment, can have negative total dipole moment and be popped out from the strong field. Evaluations show that for the tension of field 10-30 V/cm, for the scale of spottiness (size of hole) 10 μm, for the particle with their own dipole moment of tens D, dipole velocity of drift on the order of value wholly comparable with electrophoretical (Coulomb) velocity of drift and can be watched during the time characteristic for the electrophoresis of live cells . During this process cells near hole will be divided in factions, in accordance with the value of squared dipole moment.

During the process of such electrophoresis all cells with positive dipole moment, will gather in the hole. This is a defect of this approach – protein fractionation according to the dipole moment in such system causes their accumulation (focusing) in the field of strong field. Different types of cells concentrate on small distances from each other (spots of focusing for different factions are comparatively close).

To realize fractionation of cells according to the dipole moment is three options are offered .

Example 5.

The first variant is that right camera is changed by the fine capillary, in which factions will be divided. This is a sort of dipole eye dropper will contain factions divided upon their dipole characteristics. In order to avoid the agglomeration of the fractions in the capillary at the input, it is necessary to apply a small electrical field. Thereby, on electrodes an electrophoretical cell a periodic asymmetric electrical signal, with the small average floor for a period and big average square of the tension of field, is needed to be applied.

Example 6

One more variant of fractions division according their dipole characteristics is that particles, collected (focused) in the hole in Example 4, by applying variable increasing in the average amplitude field. During this event first of all particles, with the largest velocity of driftage, i.e. that particles, that have the largest ratio of an Coulomb power to dipole power, will began to leave. If an average of the field gradually, slowly increases, the hole consecutively will leave all fractions, focused in it before during dipole focusing, described in the Example 4.

Example 7

They offer to produce a "sucking out" of the most mobile cells in the field by constant electric field into the special capillary. In order to do this the area of instrument, where separation is produced, will be connected by the special capillary with the third electrode and when voltage is applied the most mobile cells will be "sucked out " from the area of separation. For the accumulation of concentrated cells special terminals were done (Fig. 31) Presence of nonlinear depending upon the electrical tension of field velocity of drift allows also to realize a current focusing of the fractions. Notice that during this focusing maximum of resolution is reached.

The next example is connected with focusing the cell fractions in the another more complicated structure.

Example 8

Consider a coniform narrowing capillary, with non-equal(corrugated) form, with the input radius 10 μm, output radius 2 μm and length 100 μm. General narrowing of the capillary such that its section is changed according the hyperbolic law. Surface of the capillary has periodic narrowing and expansions with the amplitude 0.5 μm(Fig. 30 n).

Each of the narrowing of the capillary presents itself as if there is a hole, in which focusing of particles is possible. Capillary is filled with the electrolyte and placed in the electrophoretical cell. If variable voltage with small, in contrast

with the fluctuation amplitude, average (average field <E> is 2-5% from the amplitude E_0),is applied focusing of separate fractions is possible, moreover each fraction finds its "own" hole, in which it will stop. Indeed, because section "in average" along the length of capillary is narrowed in the hyperbolic way, tension of the field "in average" linear increases. Herewith Coloumb power, that proportional to the field, grows linear, and dipole power grows in square law according to the distance from the beginning of capillary. Presence of corrugated edge of the capillary brings about to the periodic appearance of some sort of "dipole barriers" that different factions are needed to "overcome". Delay ability of such barriers grows with the distance , counted out from entry in to the capillary. For each fractions there is its own barrier, which it unable to overcome and it is focused in this place.

Example 9

This is an illustrative example of separation of several particles by the TCW method.

The system under study consisted of a gonococcal vaccine made of strains of gonococcus bacteria. The concentration of bacteria is 100/ml.

These bacteria were stained with propydium and eosine for fluorescent imaging.

The separation cell consisted of a rectangular vessel 10 cm long and 7cm wide filled a mixture of a phosphate buffer [Na_3PO_4 (0.1 M), Na_2HPO_4 (0.1M) in a water solution] and a water solution of Glycerol in 1:1 ratio. Two platinum wire electrodes were submerged at both ends of the cell.

When a variable voltage waveform as shown in Fig. 35 was supplied from the power supply to the electrodes a periodical charge wave was generated and as a result several fractions of the vaccine were separated as shown in Fig. 35. Separations of 1cm between fractions were achieved in several hours.

Example 10

In this illustrative example a single charge wave was generated by the electrolytic process for the focussing of Erythrocytes dispersed in a physiological solution. Two focussing systems were employed.

In one of them a substrate made of filter paper was wet by the physiological solution together with the dispersed red blood cells.

When a voltage was applied to the wet paper through the electrodes a sharp front of erythrocytes was observed advancing from both electrodes toward the center and finalizing in a sharp band visible in the center as seen in Fig. 36. This effect was further demonstrated by using pH indicators and visualizing the charge wave .

Claims

1. A method for moving, isolating and/or identifying particles in a sample by placing said sample in a spatially varied electrical field wherein the spatially varied electrical field is following a mathematical function selected from the group consisting of hyperbolic, parabolic functions and combinations thereof.

2. The method of claim 1 wherein the spatially varied electrical field is achieved by varying the cross-section area of the medium normal to the direction of the electrical field.

3. The method of claim 2, wherein the mathematical functions are following combinations of hyperbolic, parabolic functions obtained by linear combinations, multiplications and/or divisions of said functions.

4. The method of claim 1, wherein the medium is limited by limitation means having a shape of two converging hyperbolas.

5. The method of claim 1 to 4 combining at least two separation media having different dielectric or conductivity properties.

6. The method of any one of claims 1 to 5, wherein the spatially varied electrical field is achieved by a multielectrode arrangement and the potential between two adjacent electrodes along the medium is different.

7. The method of any one of claims 1 to 6, wherein the medium comprises a fluidum such as a gel, a liquid, a solid state, e.g. a tissue.

8. The method of any one of claims 1 to 7, wherein the electric field is changed in a stepwise form to obtain initial focusing and compression.

9. The method of any one of claims 1 to 8, wherein a steep field jump is obtained in the transition region between two gel segments, with different electrical properties.

10. The method of any one of claims 1 to 9, for separating larger particles from smaller particles which are suspended in a medium by applying a non-linear electric field across the medium,

- such that different particles will move in opposite directions or

- large particles move faster than small particles or vice versa.

11. The method of any one of claims 1 to 10, for separating larger particles from smaller particles, the method comprising the following steps: suspending the particles to be separated in gel medium, wherein the gel has a variable cross section geometry, applying a non linear electric fields across the medium causing the particles to travel through the medium in the forward direction.

12. The method of any one of claims 1 to 11, wherein the electrical field is varied time dependently, e.g. periodically for focussing, i.e. immobilization of particles in the separation medium.

13. The method of any one of claims 1 to 12, wherein the spatially varied electrical field is achieved by producing a complex separation sequence and media by generating a continuous path of separation gel made up from segments with different electrical properties and by applying different electric potentials to each segment or by generating different electric fields in each segment.

14. The method of any one of claims 1 to 13, wherein the non uniform field is generated in a capillary.

15. The method of any one of claims 1 to 14, wherein the capillary is filled with segments of gel with different electric properties.

16. The method of any one of claims 1 to 15, wherein the non-uniform field is generated by segments of polymer tubes with different diameter placed in the capillary.

17. The method of any one of claims 1 to 18, wherein non polymerized gel is applied to the surface of the substrate by means of a special pen-like

dispenser filled with gel, the pen being a part of a plotter operated by a special software.

18. The method of any one of claims 1 to 19, wherein the gel sequence is assembled from segments of gel cut to special shape by a software driven cutting machine.

19. The method of any one of claims 1 to 10 and/or 17 or 18, wherein the medium for separation is on a chip and/or the method is performed by means of a micro-fluidic system.

20. The method of any one of claims 1 to 19 in combination with another electrophoretic method, such as the temperature gradient electrophoresis.

21. A separation medium having a first end portion and a second end portion and a continually varying cross-section wherein the first end portion has a larger cross-section than the second end portion.

22. The separation medium according to claim 21 wherein the continually varying cross-section varies according to a hyperbolic function.

23. The separation medium according to one of the claims 21 or 22, wherein the medium is arranged in a means for supporting the medium such as glass plates, wedges, and the like.

24. A capillary having a first end portion and a second end portion and a continually varying cross-section wherein the first end portion has a larger cross-section than the second end portion to form a tapered capillary.

25. The capillary according to claim 24 wherein the continually varying cross-section varies according to a hyperbolic function.

26. An assembly of at least two capillaries according to one of the claims 21 or 22, wherein the second end portion of a first capillary of claim 24 or 25 is directed towards the first end portion of a second capillary of claims 25 or 26.

27. The assembly of claim 25, wherein the at least two capillaries are tightly joined to each other.

28. The capillary according to claims 24 or 25 or the assembly according to claims 26 or 27 filled with a separation medium such as a gel.

29. The capillary according to claims 24 or 25 or the assembly according to claims 26 or 27 consisting of glass or artificial resins such as polymethylmethacrylates PMMA.

30. An electrophoresis apparatus for performing the method of any of the claims 1 to 21 comprising a first electrode means comprising a cathode and an anode, means for supplying a non-uniform and time dependent, in particular periodical, electric field, and a separation medium according to claim 21, such as a gel, means for supporting the medium in a geometry of varying cross-section during use of the apparatus, the anode and cathode are disposed at respective opposed edges of the separation medium to produce a polarity electric field in the plane of the separation medium.

31. The apparatus of claim 30 having means for applying designed electrical fields to the electrode means. The apparatus according to any one of claims 30 or 32 having additionally at least one reference electrode.

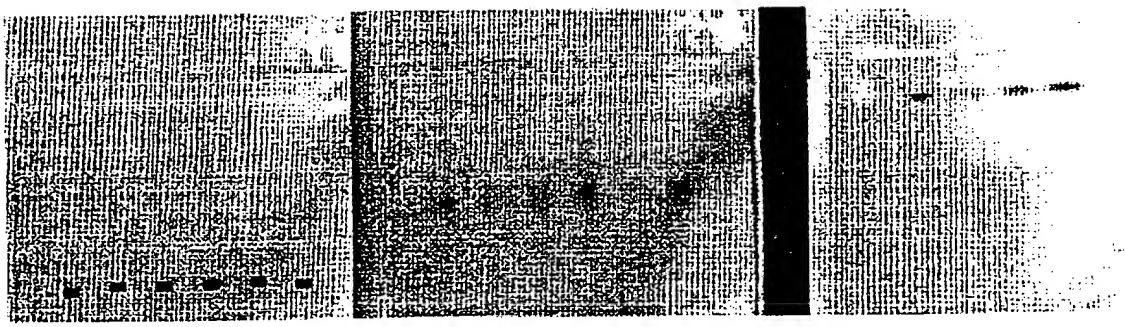

Fig.1

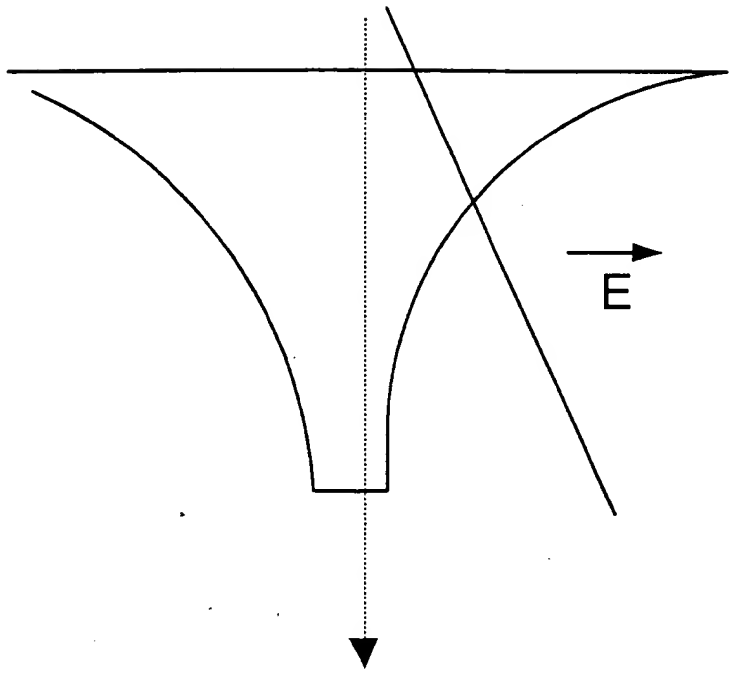

Fig.2

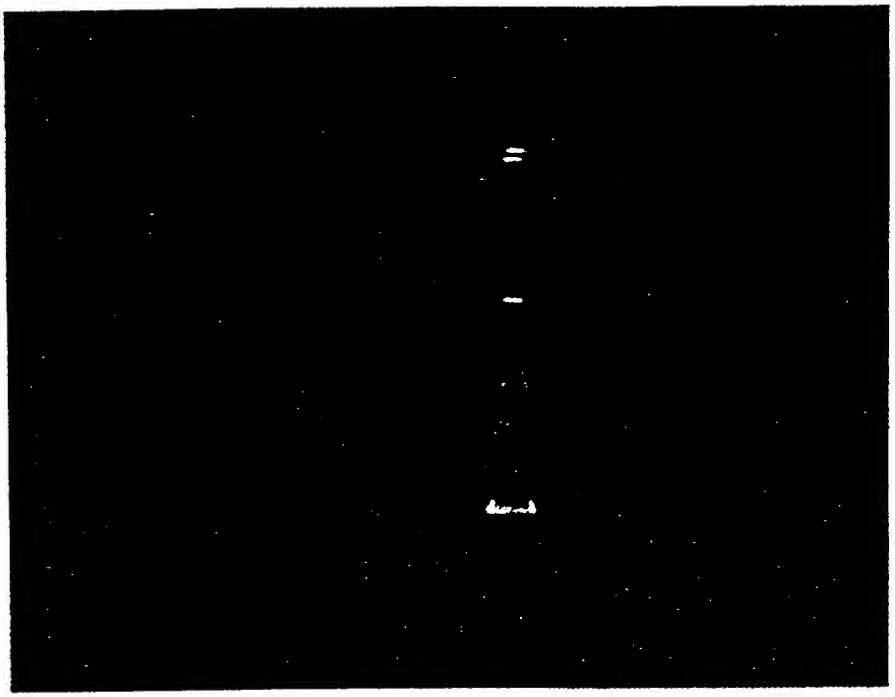

Fig.3

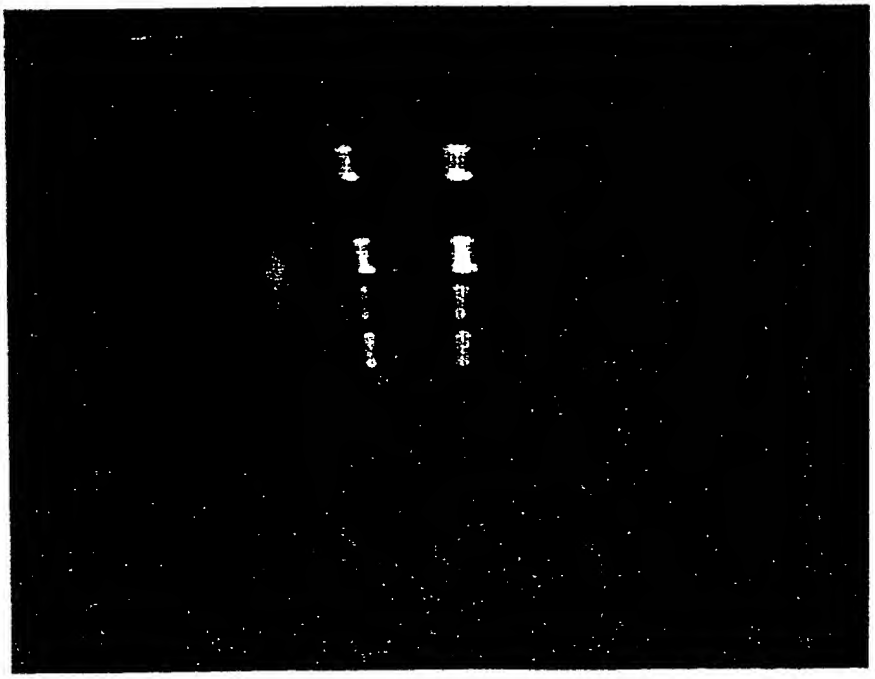

Fig.4

Fig.5

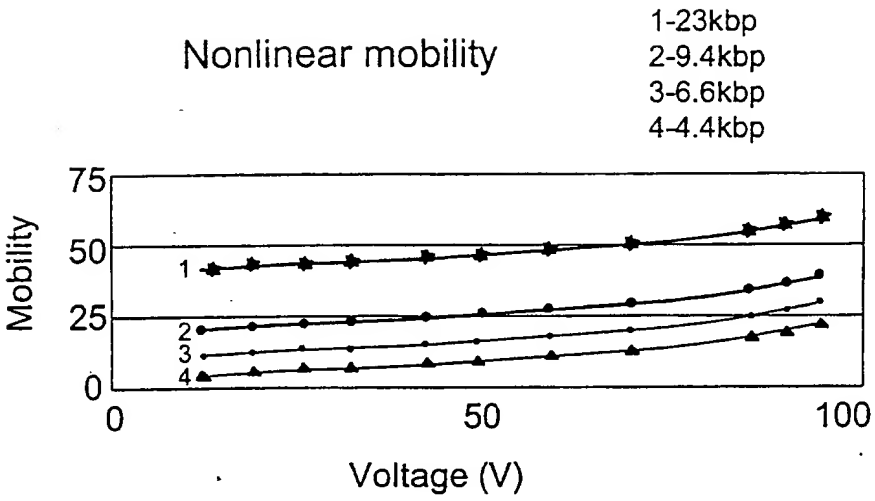
Nonlinear mobility
1-23kbp
2-9.4kbp
3-6.6kbp
4-4.4kbp
Mobility
75
50
25
0
0
50
100
Voltage (V)
1
2
3
4

Fig.6

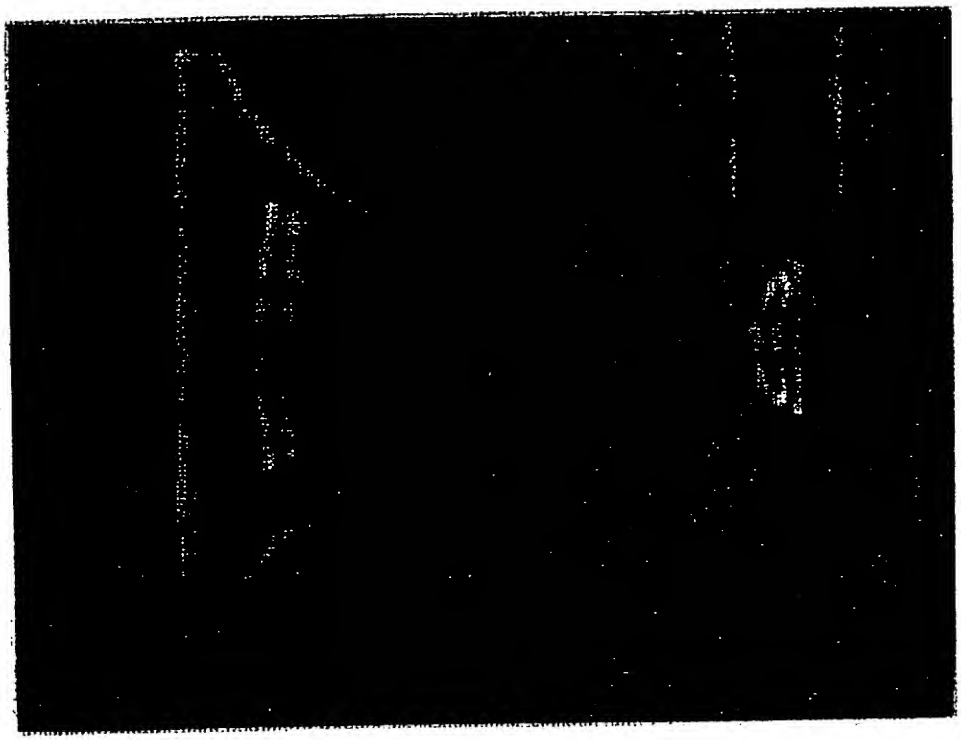

Fig.7

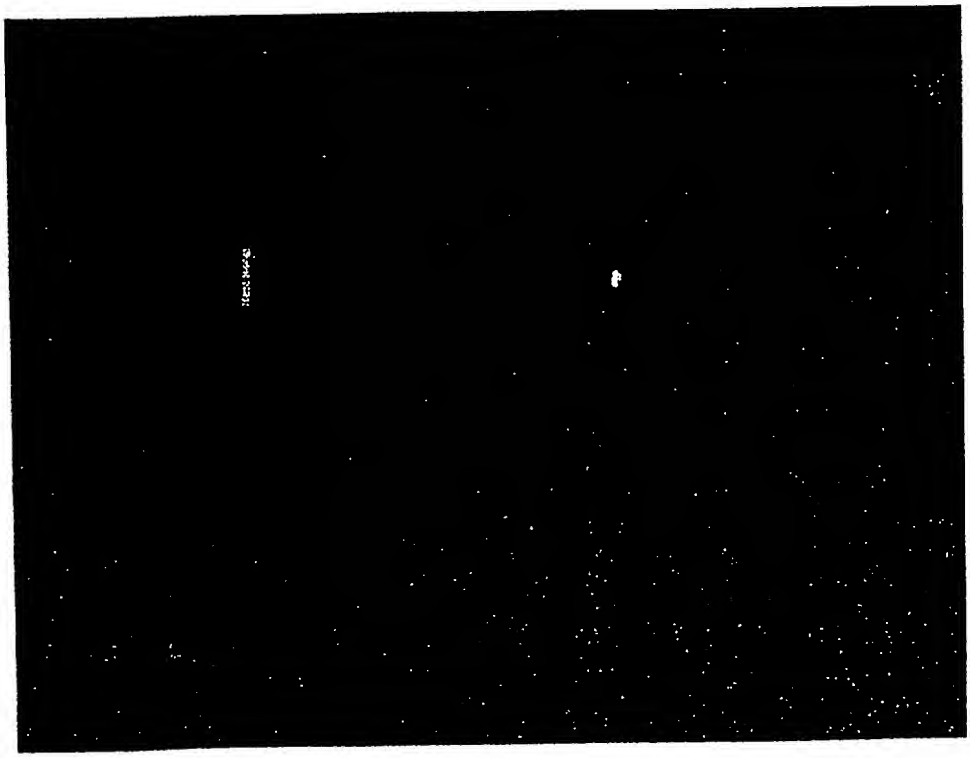

Fig.8

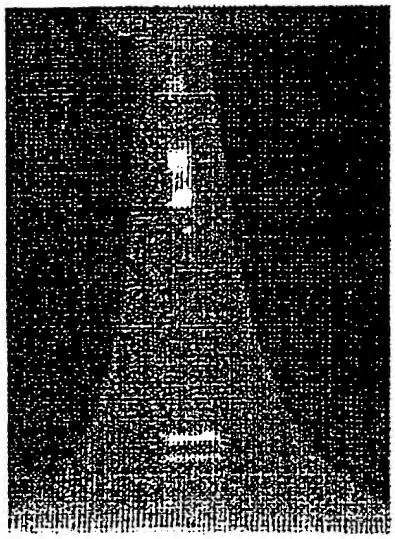

Fig.9

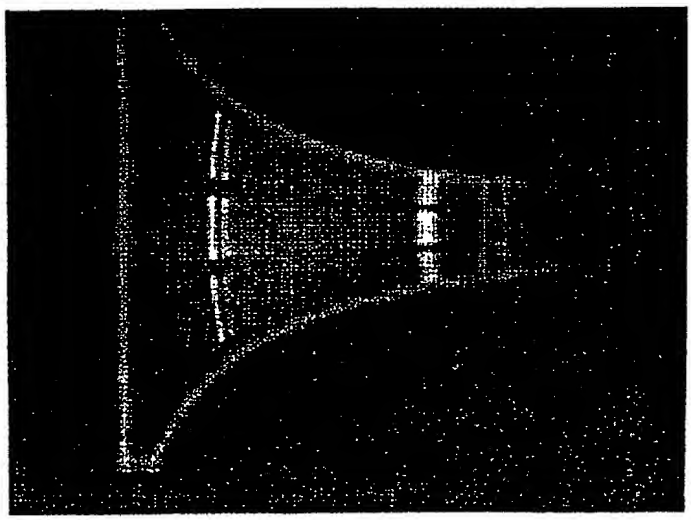

Fig.10

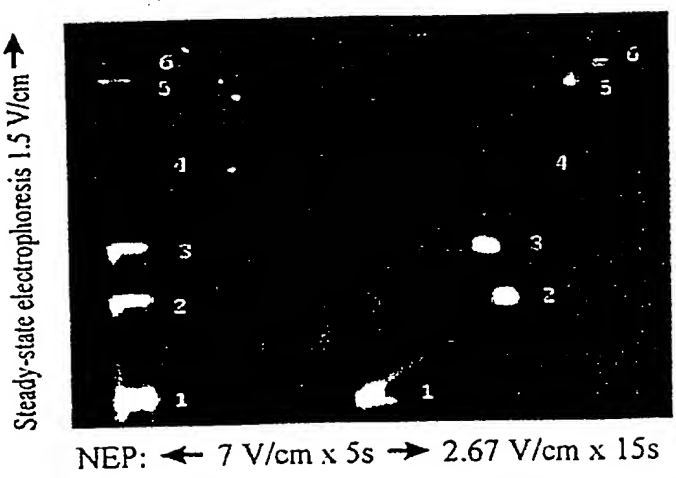
Steady-state electrophoresis 1.5 V/cm
6
5
4
3
2
1
NEP: ← 7 V/cm x 5s → 2.67 V/cm x 15s

Fig.11

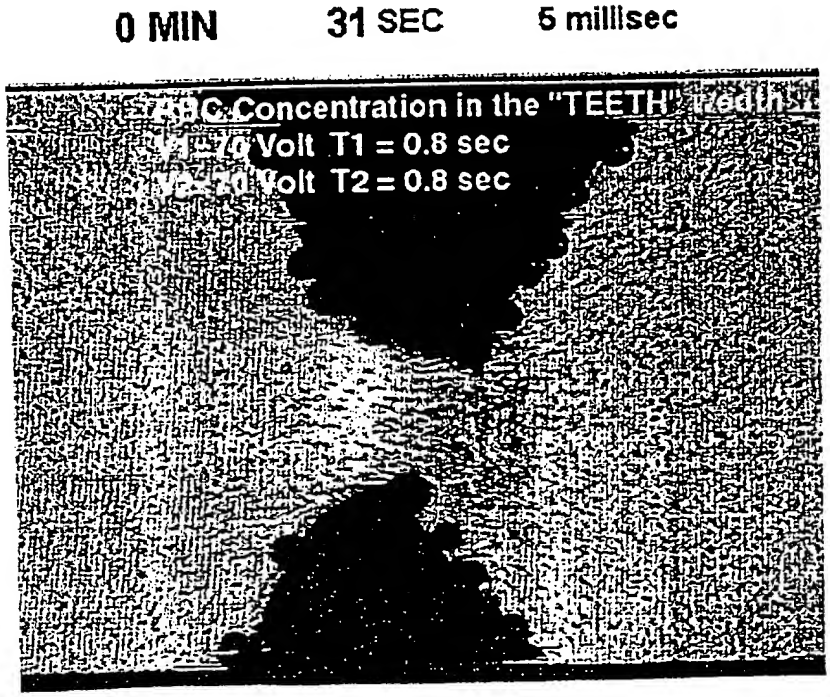
0 MIN
31 SEC
5 millisec
Volt T1 = 0.8 sec
Volt T2 = 0.8 sec

Fig.40

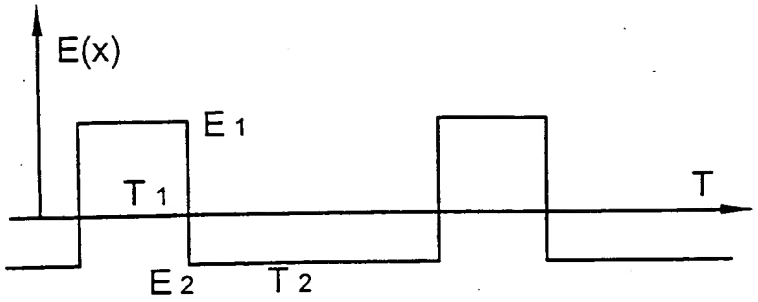

Fig.12

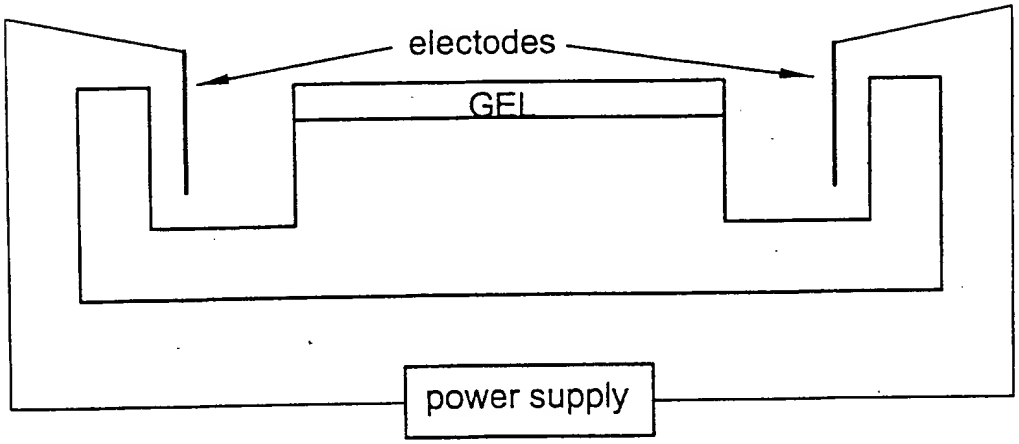

Fig.13

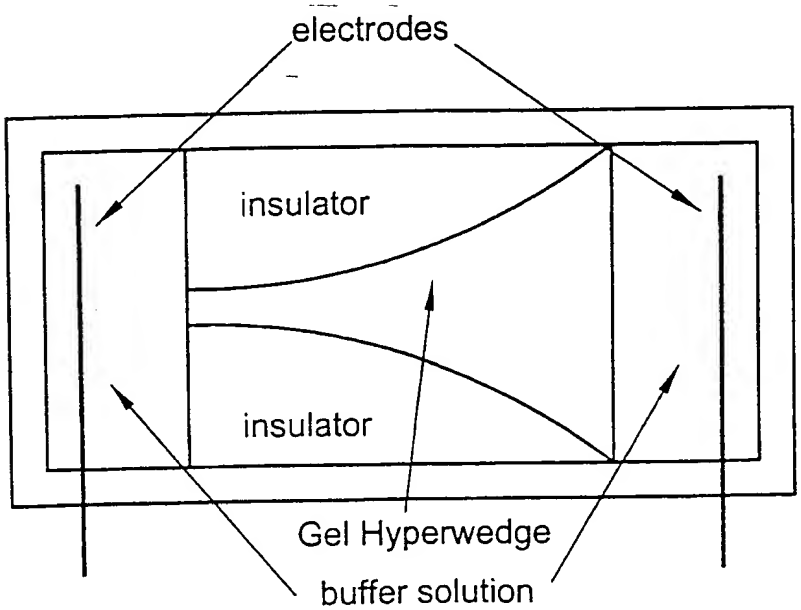

Fig.14

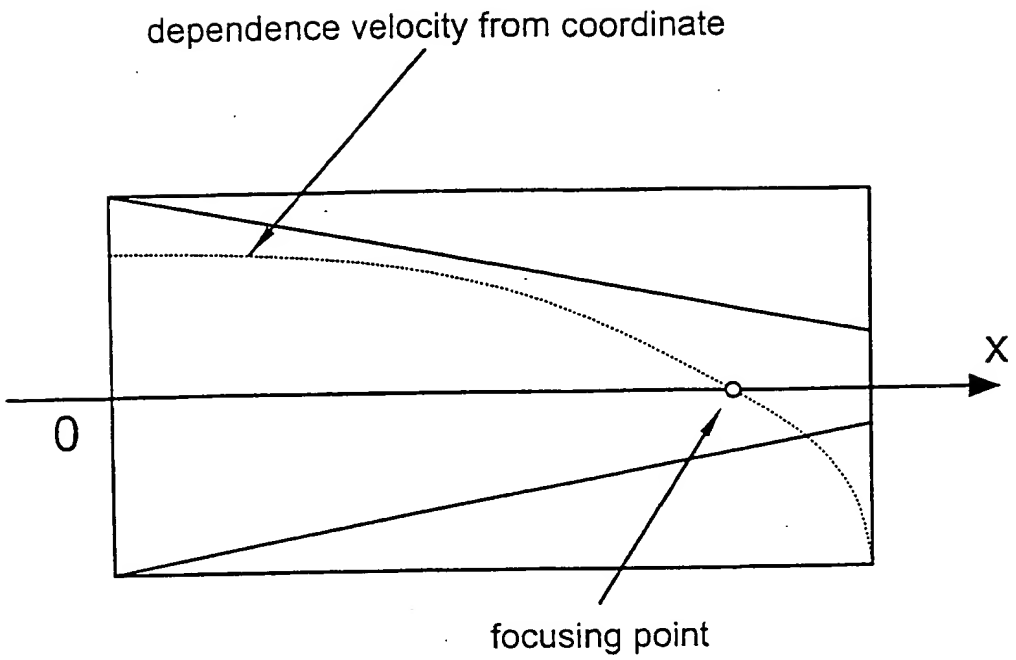
dependence velocity from coordinate
X
0
focusing point

Fig.15

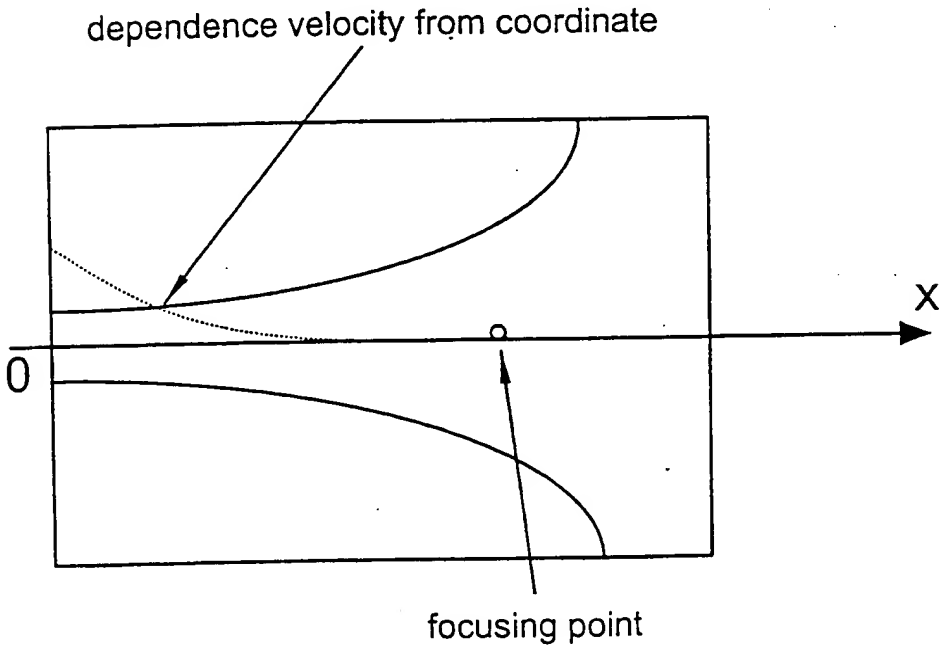
dependence velocity from coordinate
X
0
focusing point

Fig.16

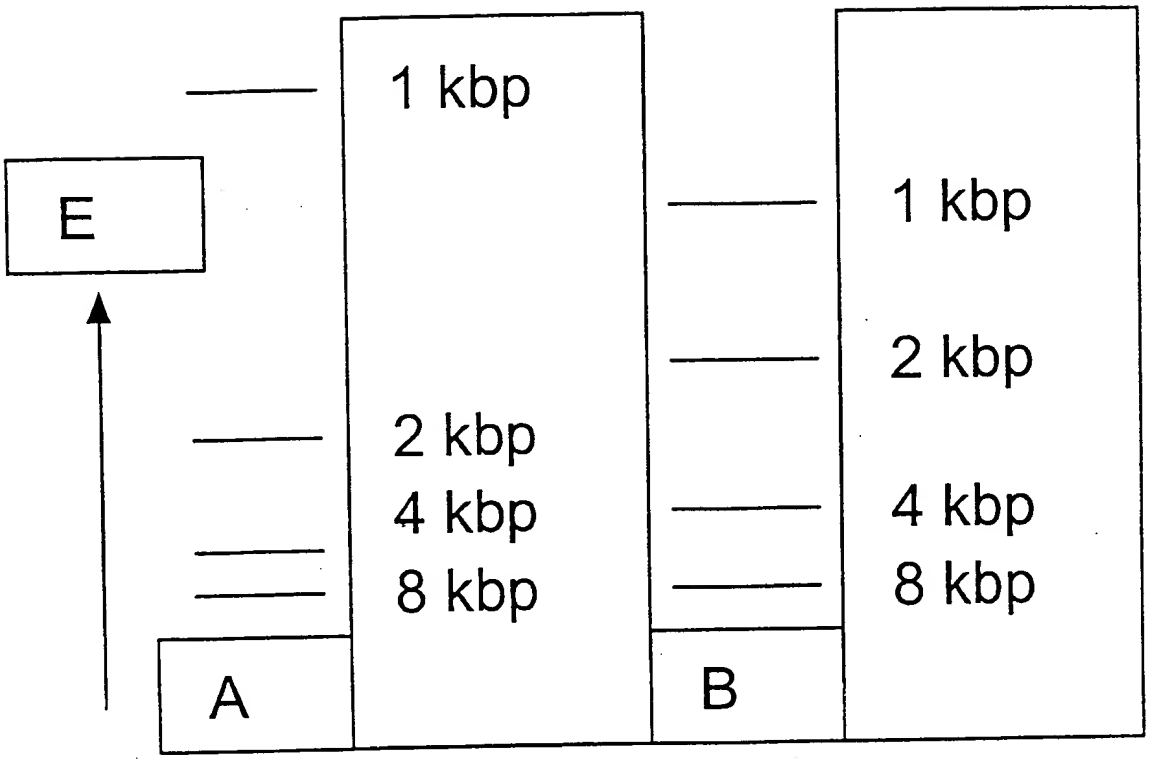

Fig.17

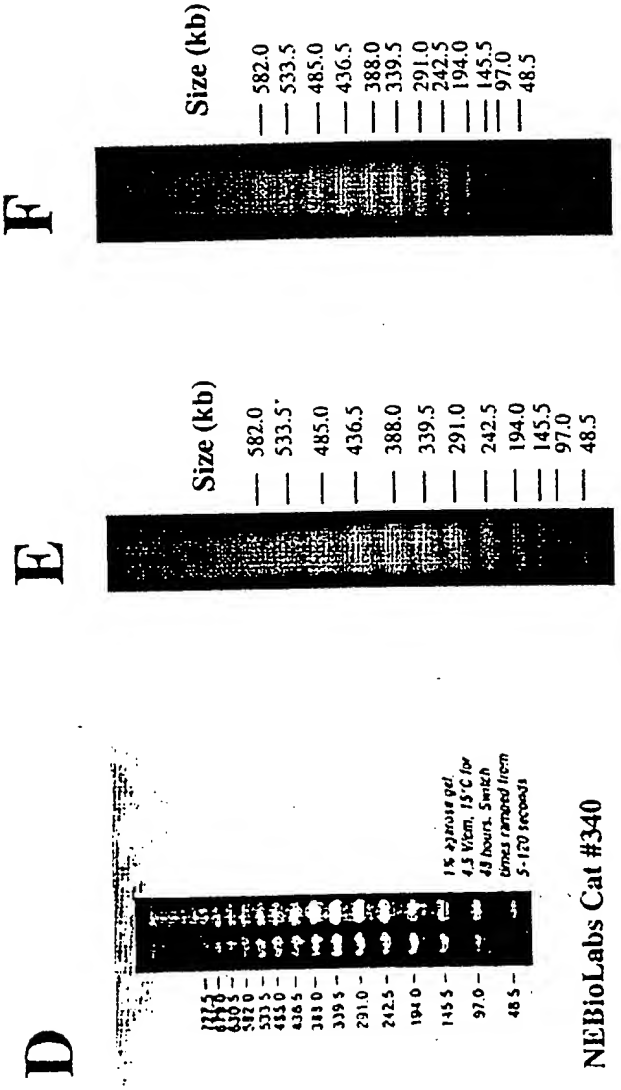
D
E
F
Size (kb)
582.0
533.5
485.0
436.5
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5
1% agarose gel
4.5 V/cm, 15°C for
48 hours. Switch
times ramped from
5-120 seconds
NEBioLabs Cat #340
48 hrs.
3.5 hrs.
3.5 hrs.

Fig.18

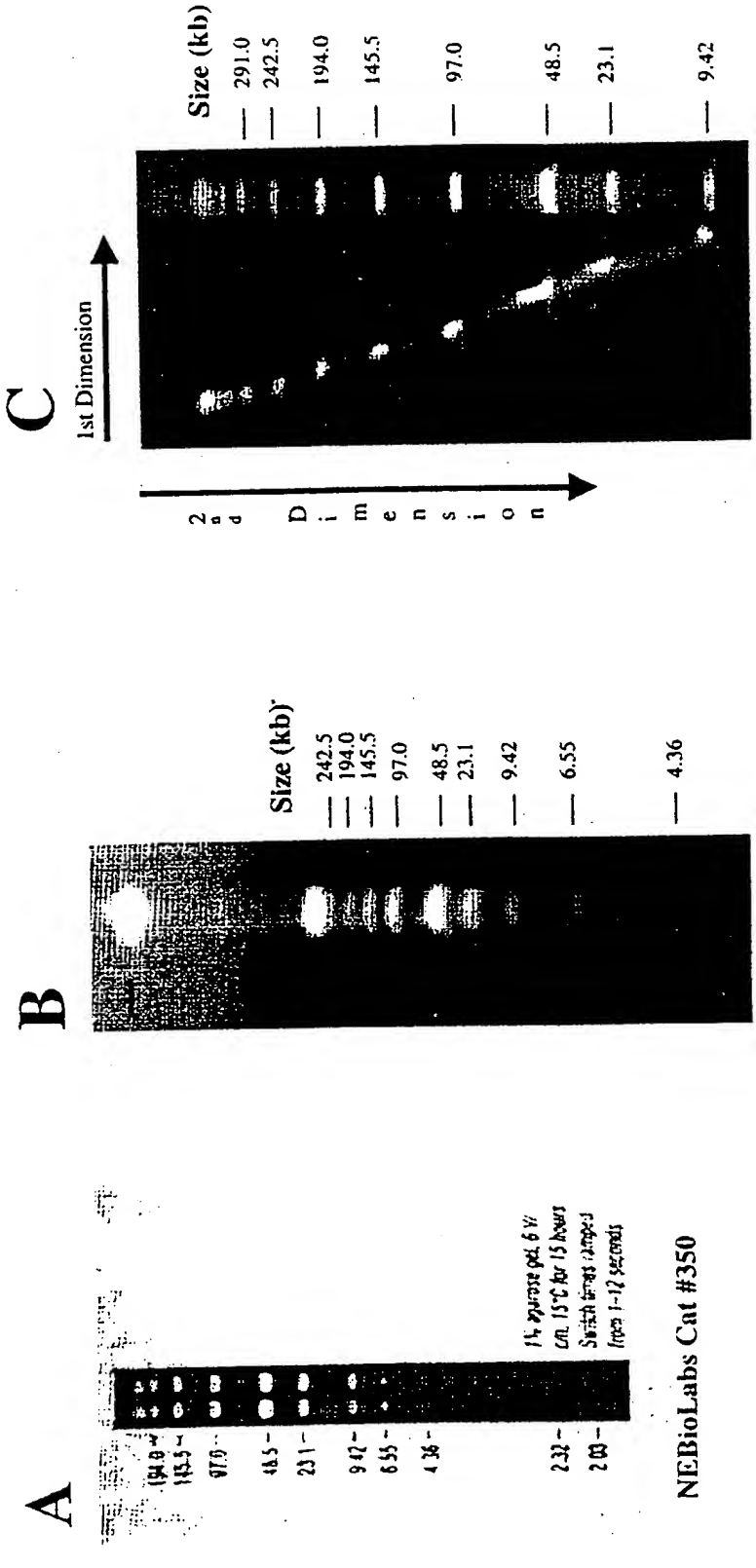
A
NEBioLabs Cat #350
15 hrs.
B
Size (kb)
242.5
194.0
145.5
97.0
48.5
23.1
9.42
6.55
4.36
2 hrs.
C
1st Dimension
2nd Dimension
Size (kb)
291.0
242.5
194.0
145.5
97.0
48.5
23.1
9.42

Fig.19

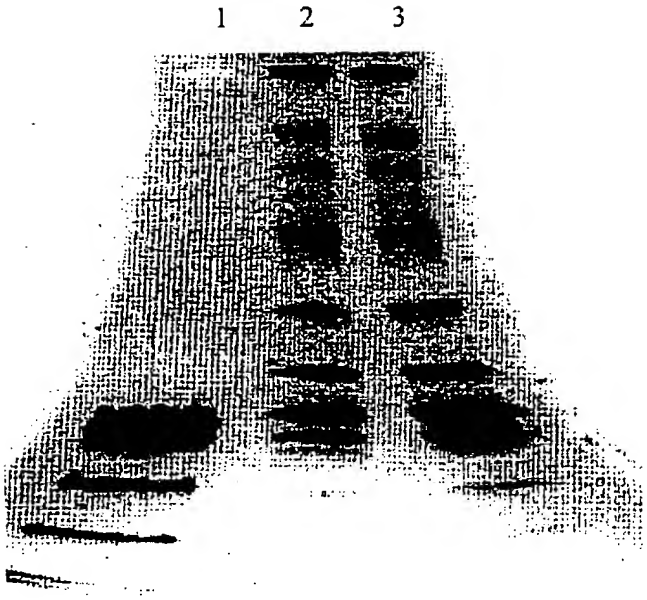

Fig.20

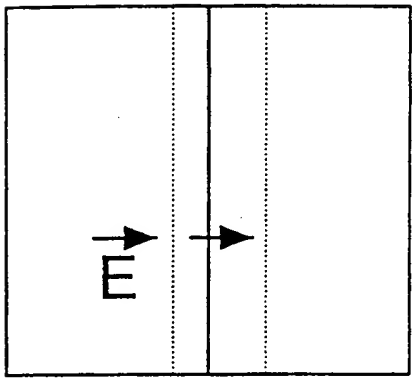

Fig.21

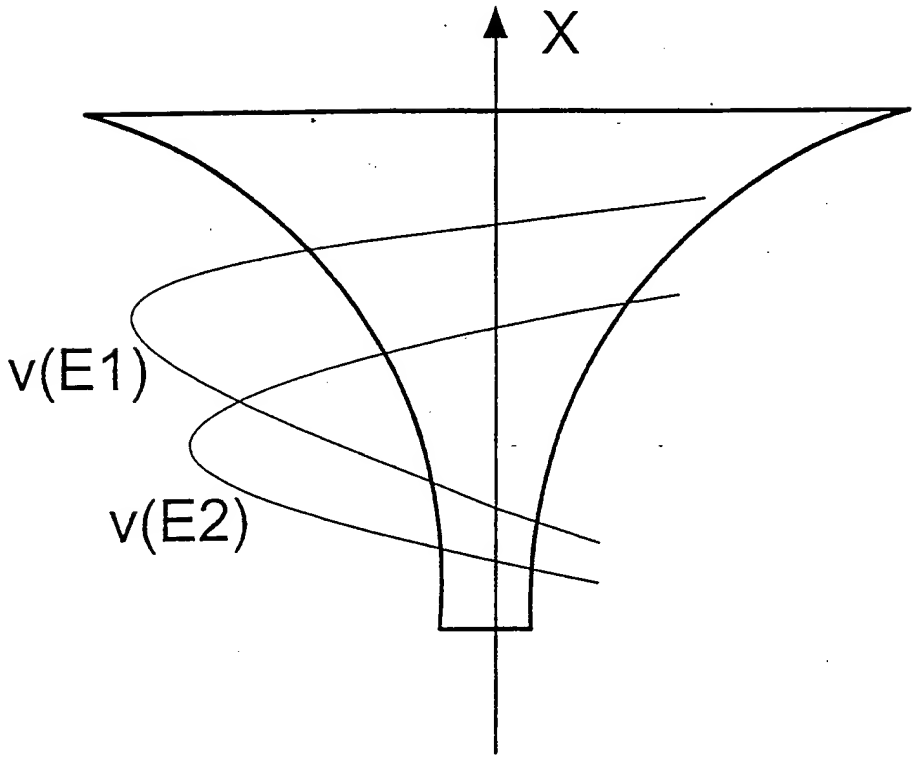

Fig.22

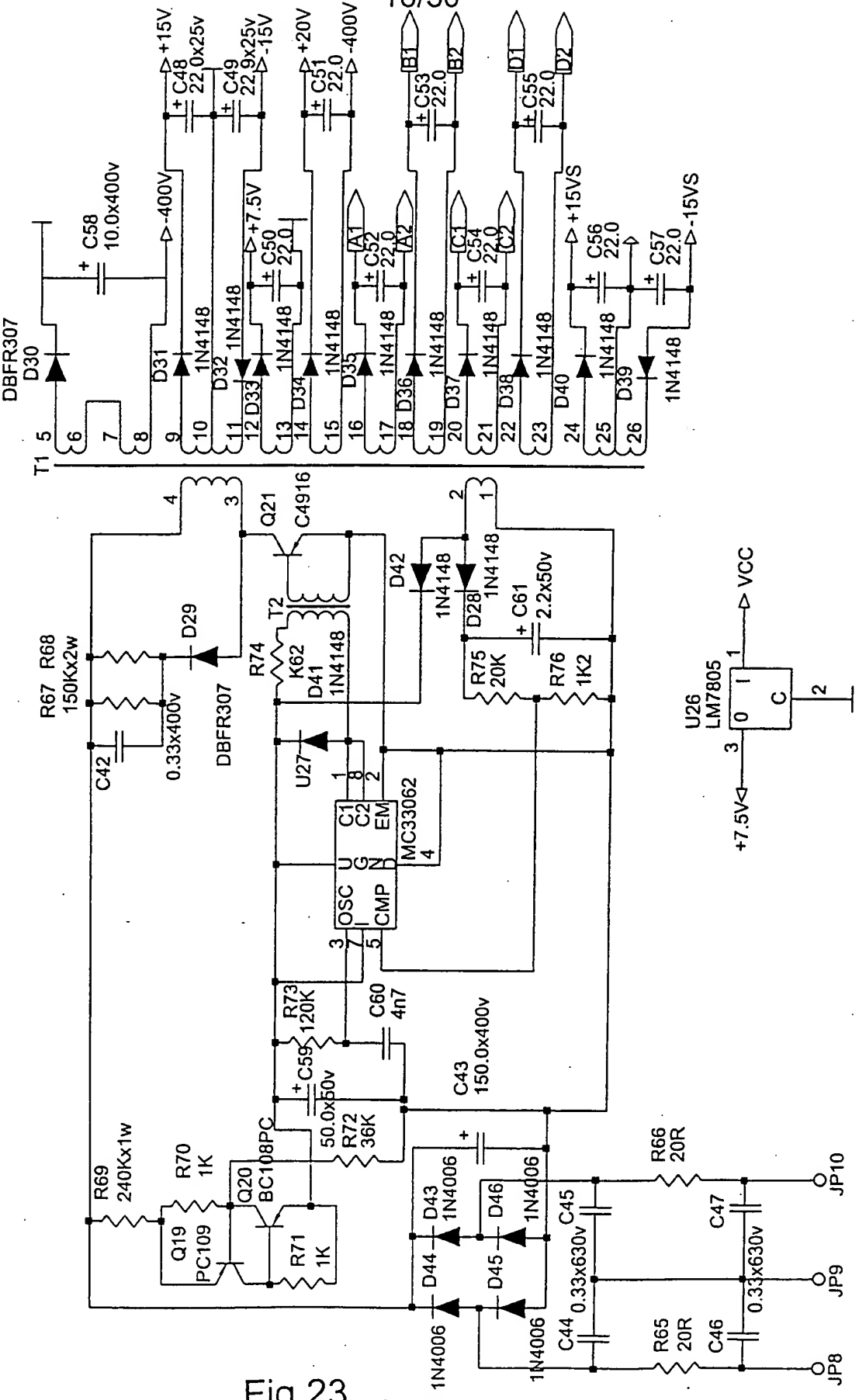
T1
DBFR307
D30
C58 10.0x400v
-400V
+15V
C48 22.0x25v
C49 22.9x25v
-15V
+20V
C51 22.0
-400V
B1
B2
D1
D2
C53 22.0
C55 22.0
+15VS
-15VS
C56 22.0
C57 22.0
D31 1N4148
D32 1N4148
+7.5V
C50 22.0
D33
D34 1N4148
D35 1N4148
A1
A2
C52 22.0
D36 1N4148
C1
C2
C54 22.0
D37 1N4148
D38 1N4148
D40 1N4148
D39 1N4148
Q21 C4916
D42 1N4148
D28 1N4148
C61 2.2x50v
R67 R68 150Kx2w
D29
DBFR307
R74
T2
K62
D41 1N4148
R75 20K
R76 1K2
C42
0.33x400v
U27
MC33062
C1
C2
EM
GND
OSC
CMP
U26 LM7805
VCC
+7.5V
R73 120K
C60 4n7
C59 50.0x50v
C43 150.0x400v
R72 36K
R69 240Kx1w
R70 1K
Q20 BC108PC
Q19 PC109
R71 1K
D43 1N4006
D46 1N4006
D44 1N4006
D45 1N4006
C45 0.33x630v
C44 0.33x630v
R66 20R
R65 20R
C47 0.33x630v
C46 0.33x630v
JP10
JP9
JP8

Fig.23

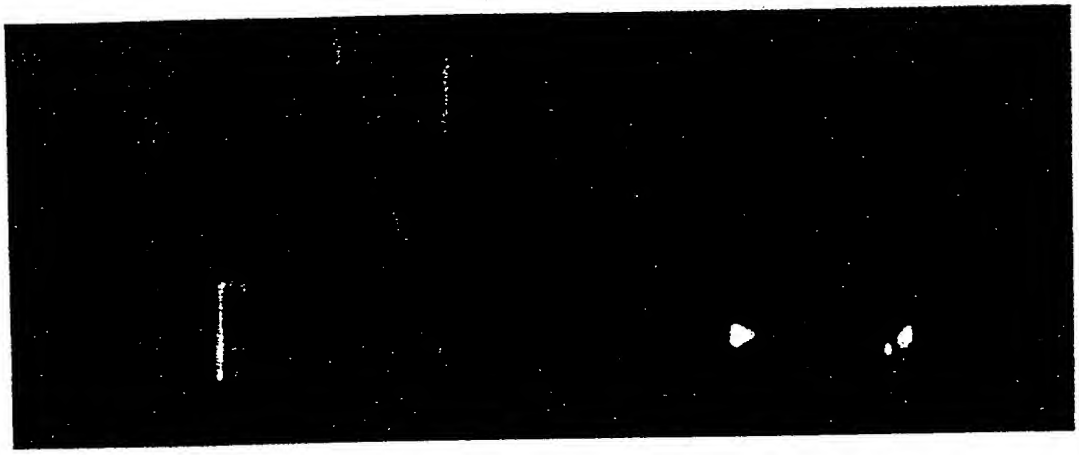

Fig.24

Fig.25

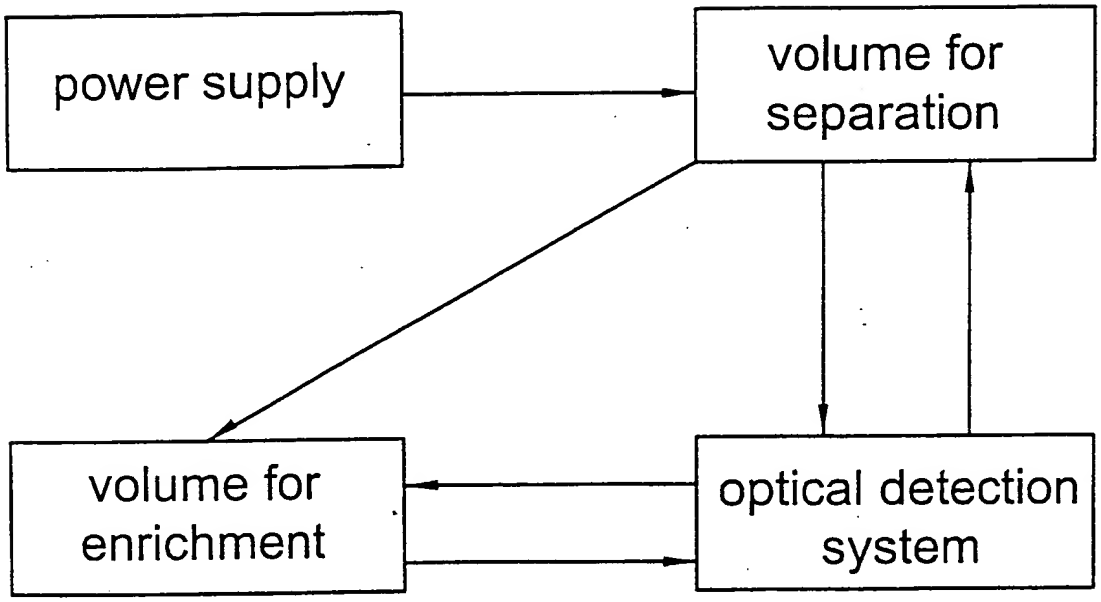
power supply
volume for separation
volume for enrichment
optical detection system

Fig.26

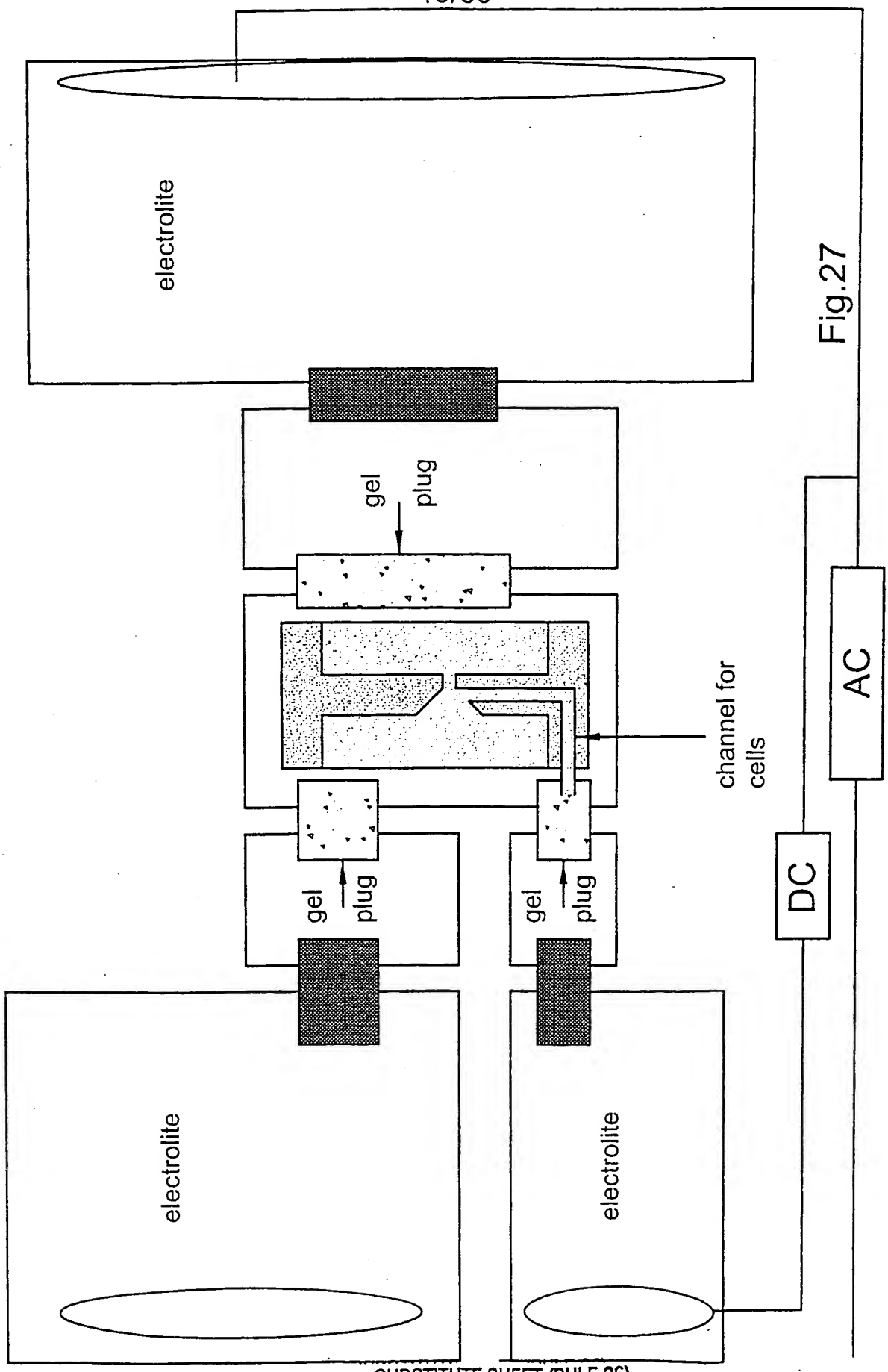
electrolite
gel
plug
channel for cells
AC
DC
gel
plug
gel
plug
electrolite
electrolite

Fig.27

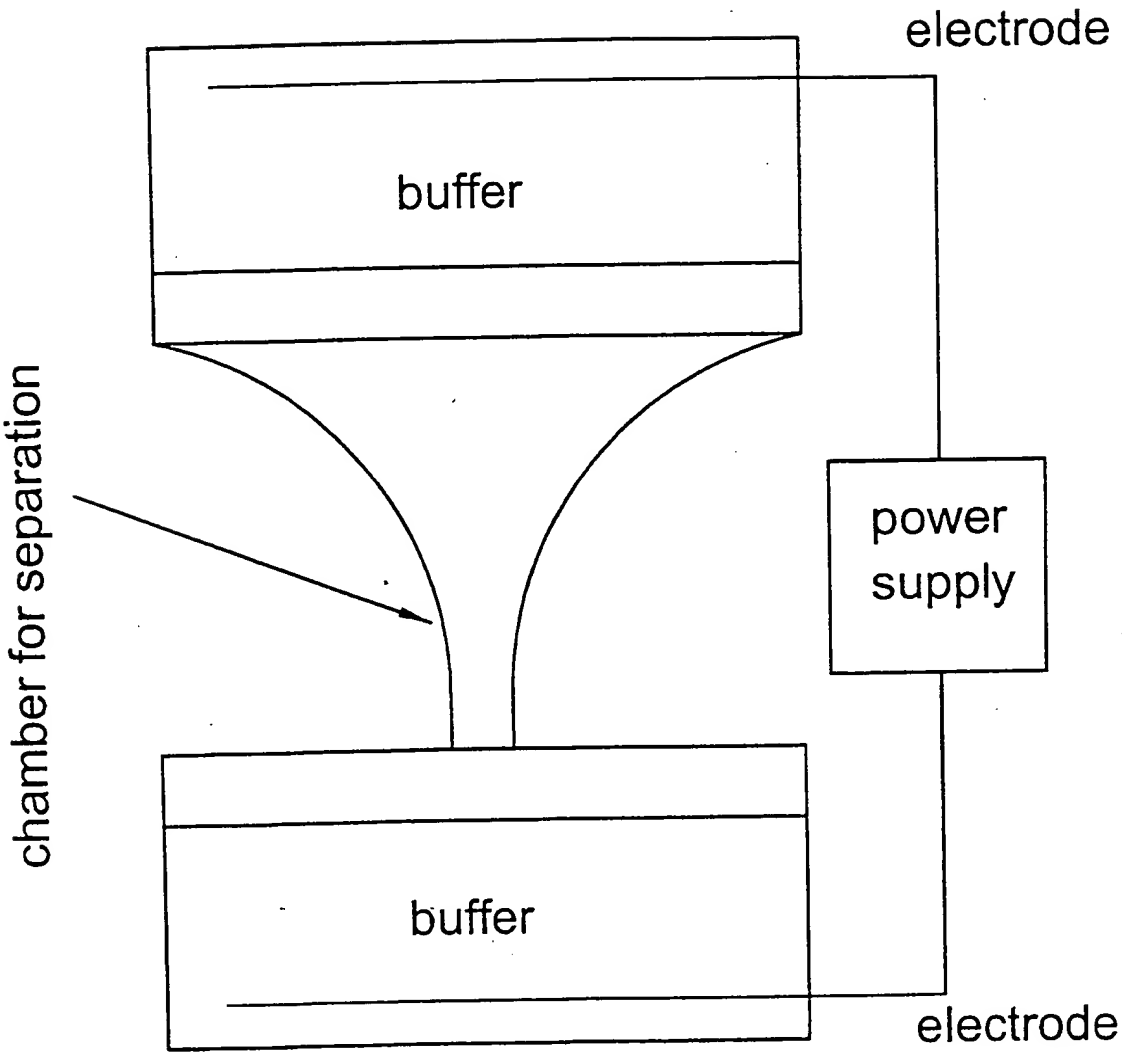
electrode
buffer
chamber for separation
power supply
buffer
electrode

Fig.28

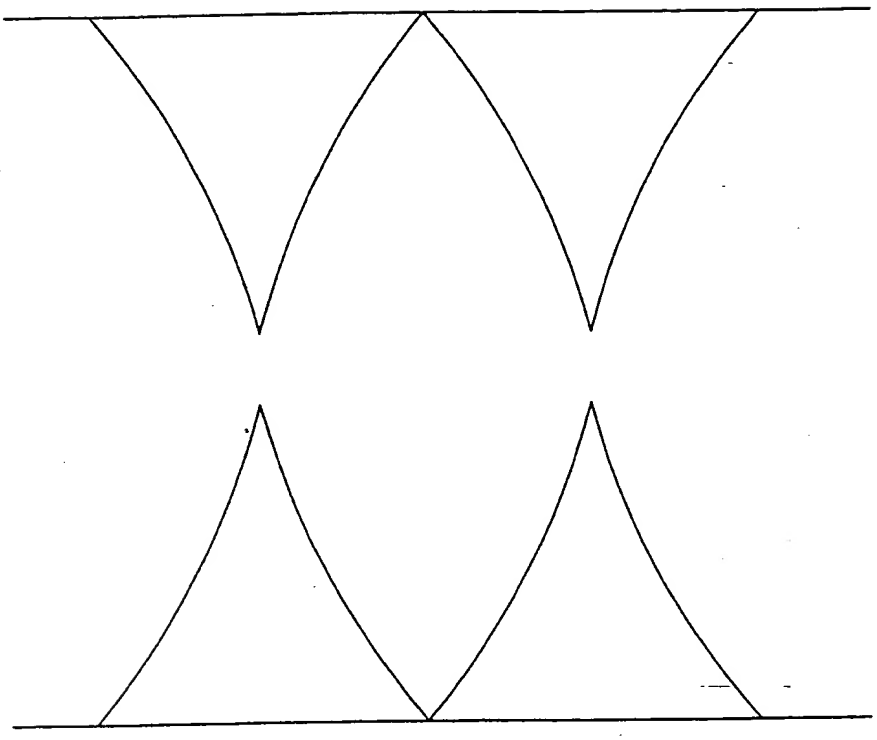

Fig.29

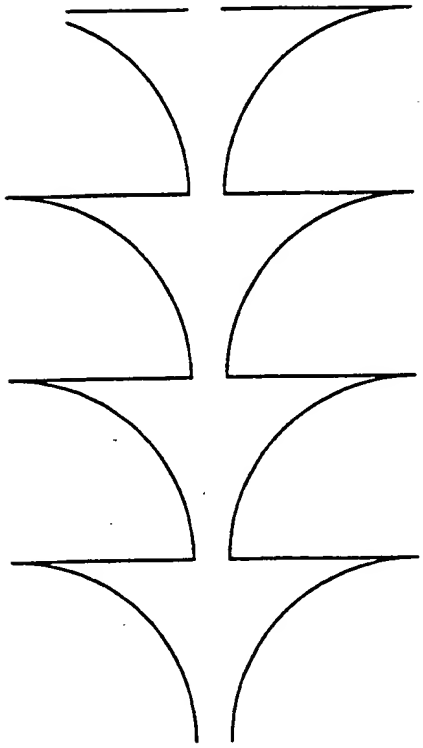

Fig.30 a

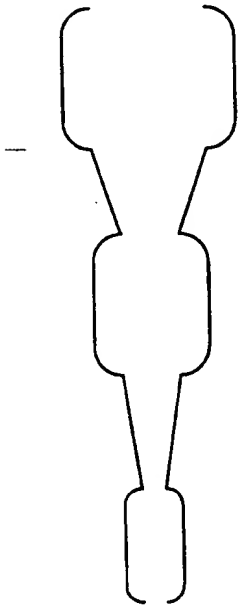

Fig.30 b

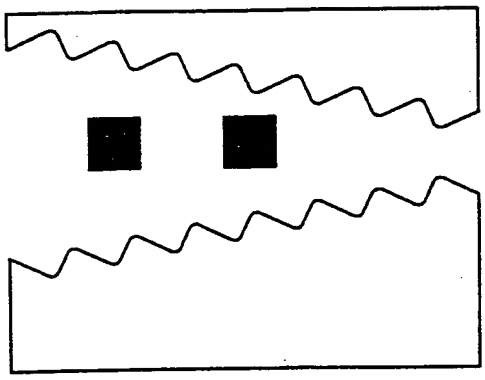

Fig.30 c

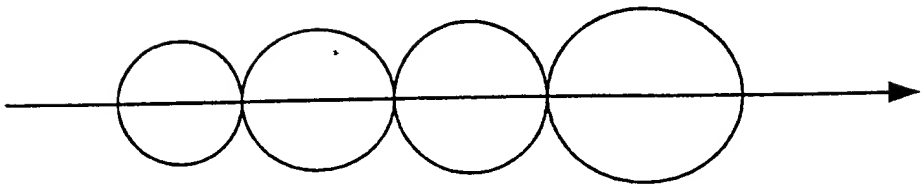

Fig.30 d

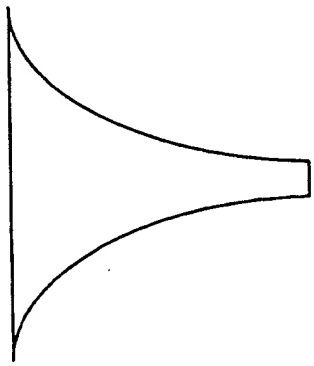

Fig.30 g

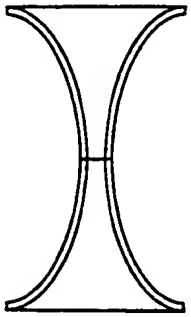

Fig.30 h

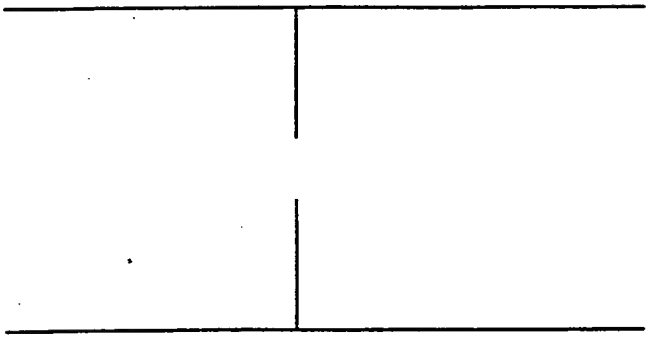

Fig.30 i

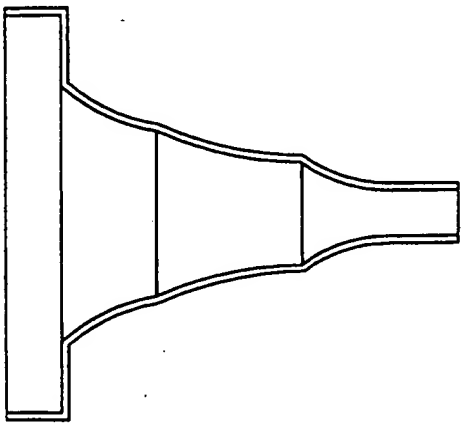

Fig.30 j

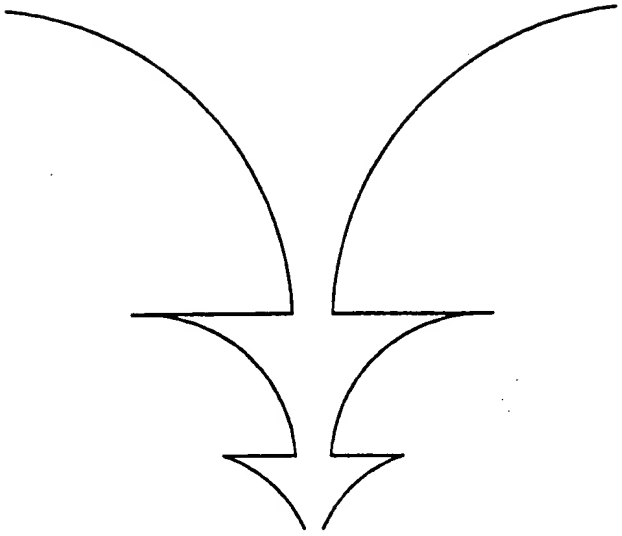

Fig.30 n

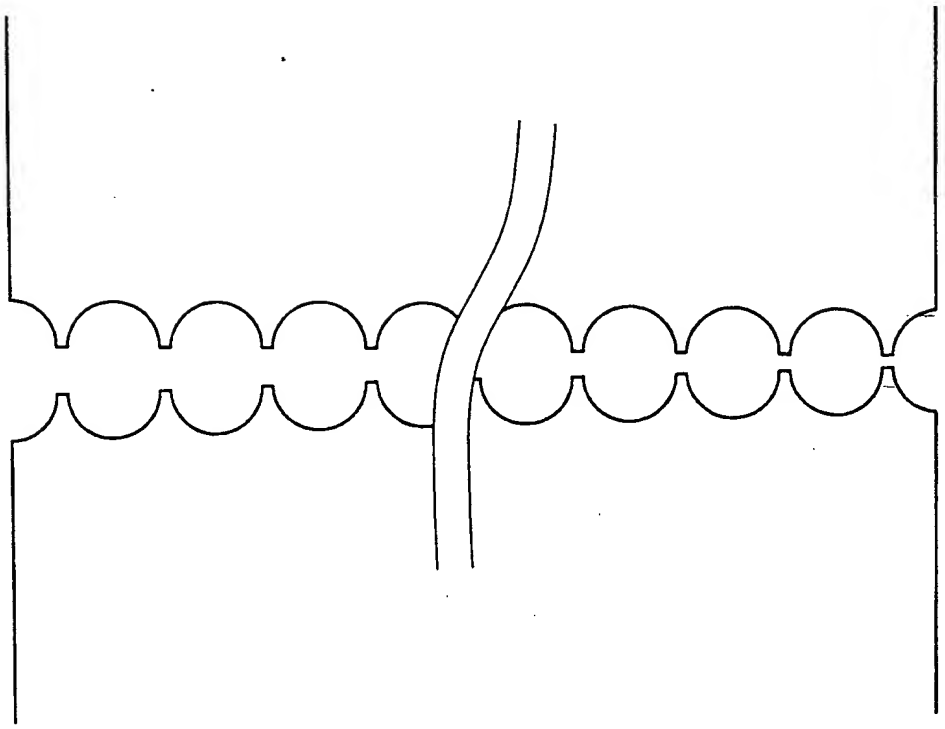

Fig.30 o

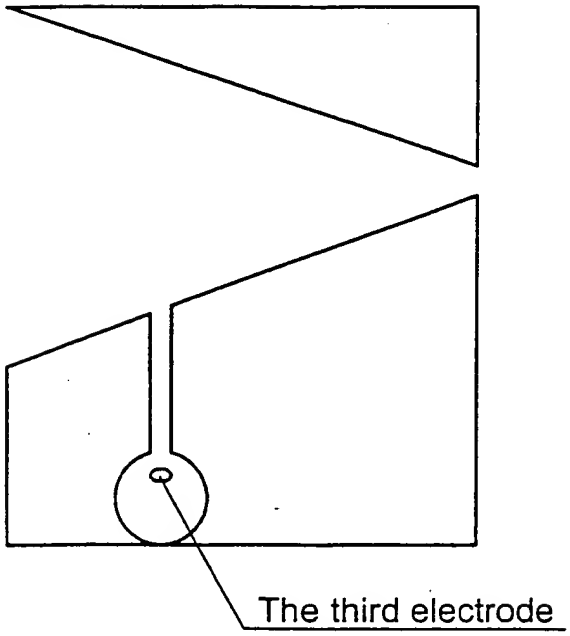

Fig.31

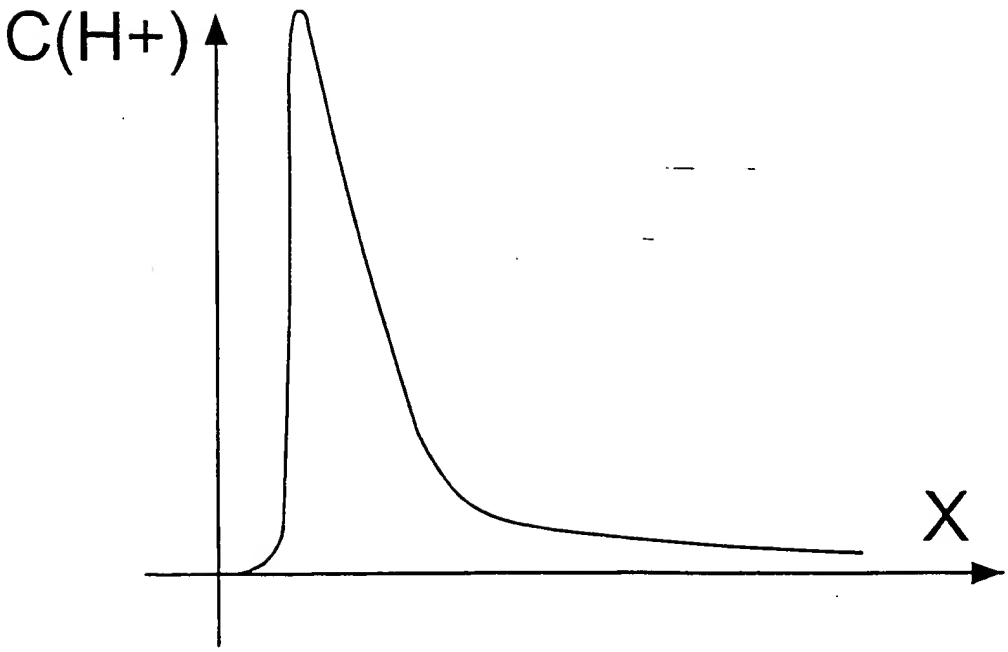

Fig.32

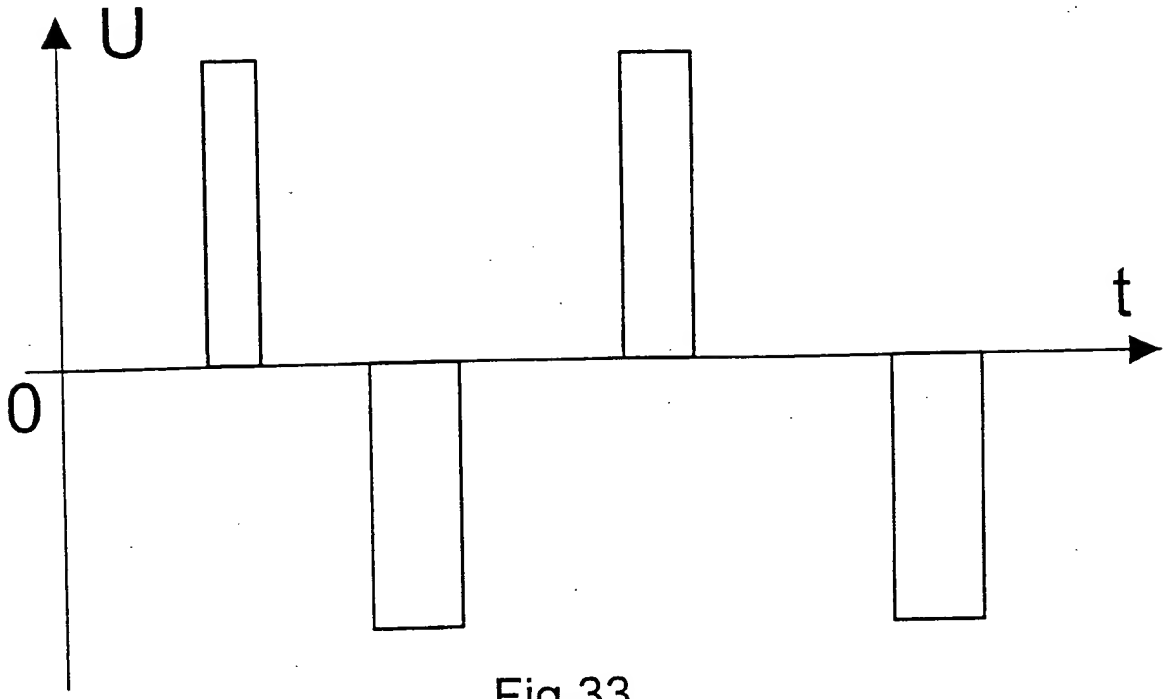

Fig.33

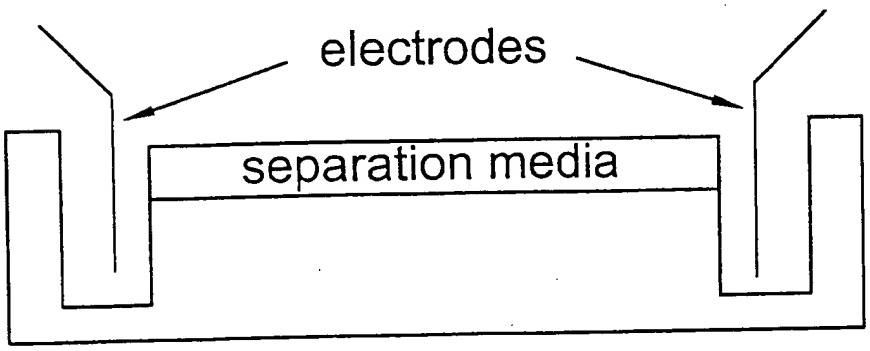

Fig.34

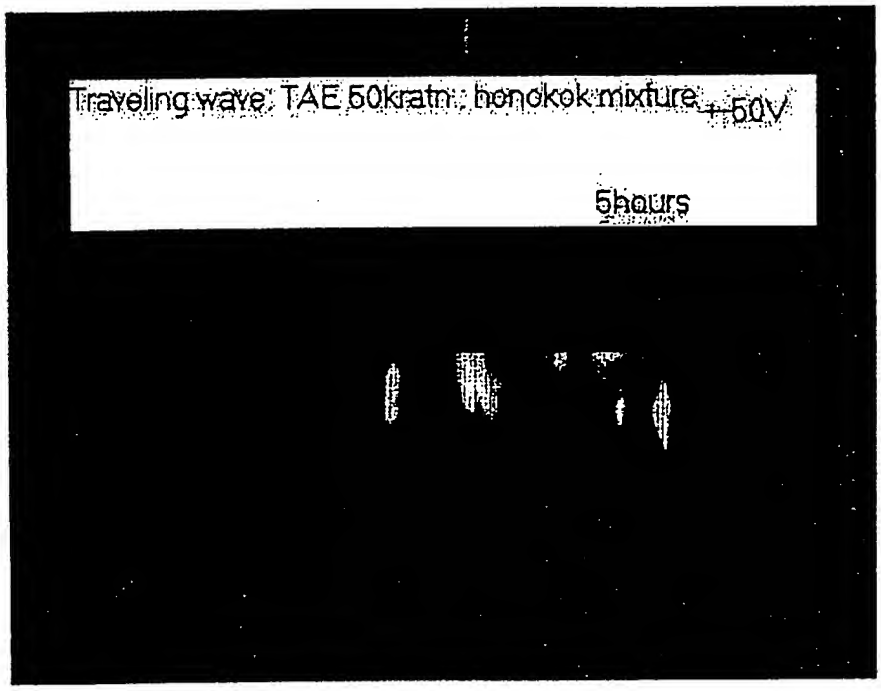
Traveling wave: TAE 50kratn : honokok mixture +50V
5hours

Fig.35

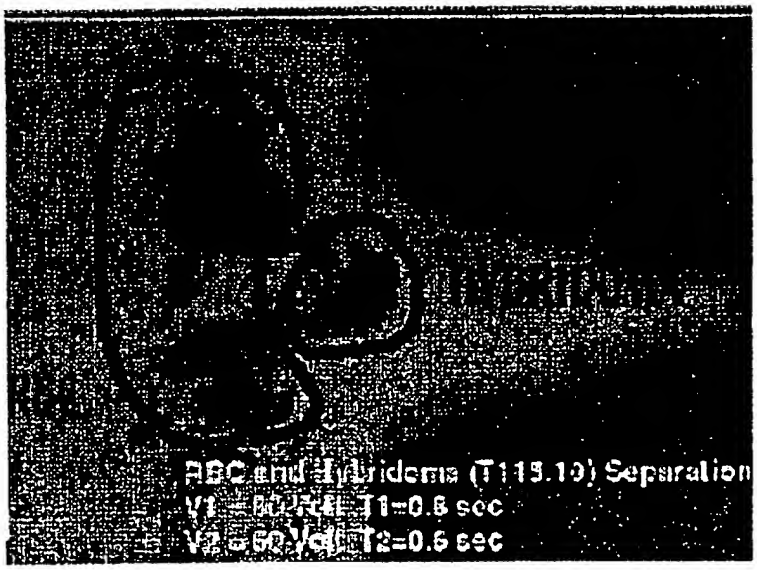
RBC and Hybridoma (T115.19) Separation
V1 = 50 Vdc T1=0.6 sec
V2 = 50 Vdc T2=0.6 sec

Fig.36

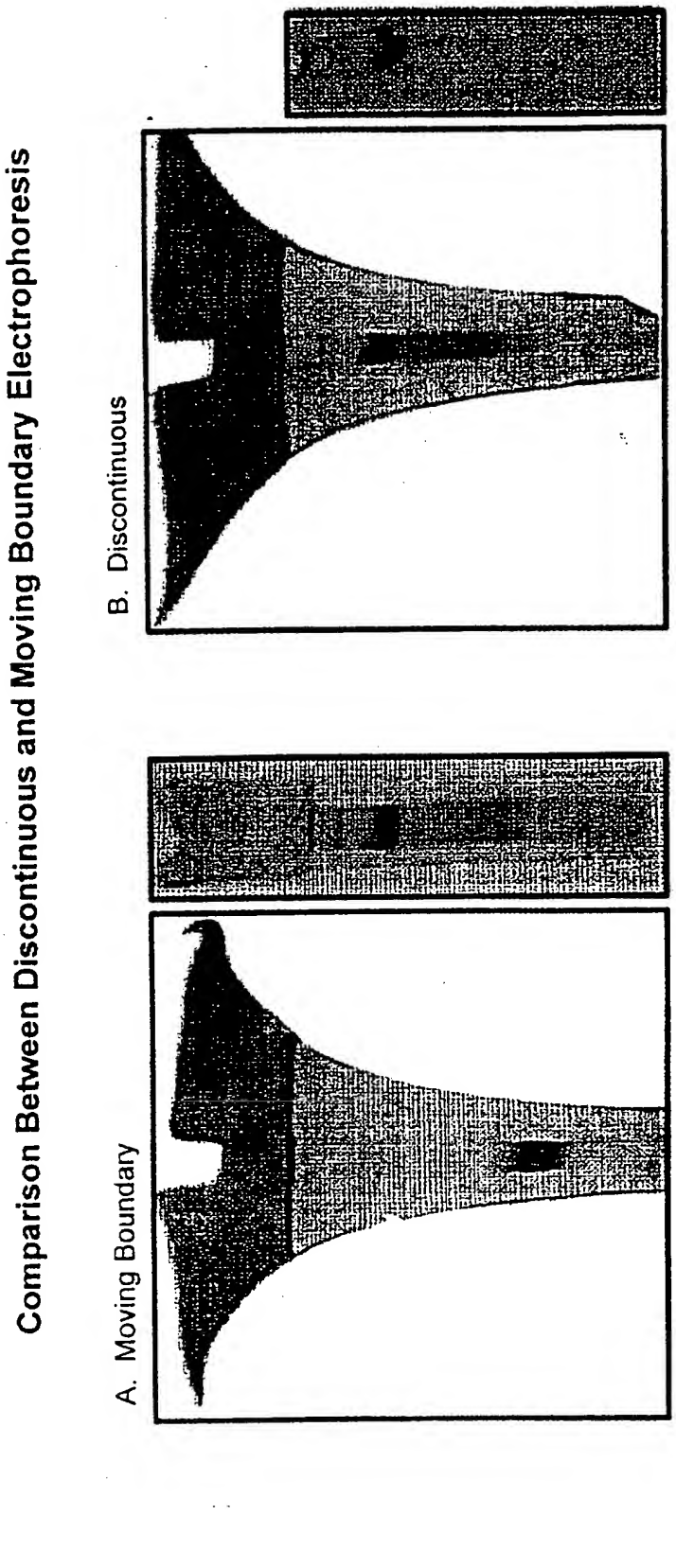
Comparison Between Discontinuous and Moving Boundary Electrophoresis
A. Moving Boundary
B. Discontinuous
Time(min):
45
45
45
45

Fig.37

Fig.38

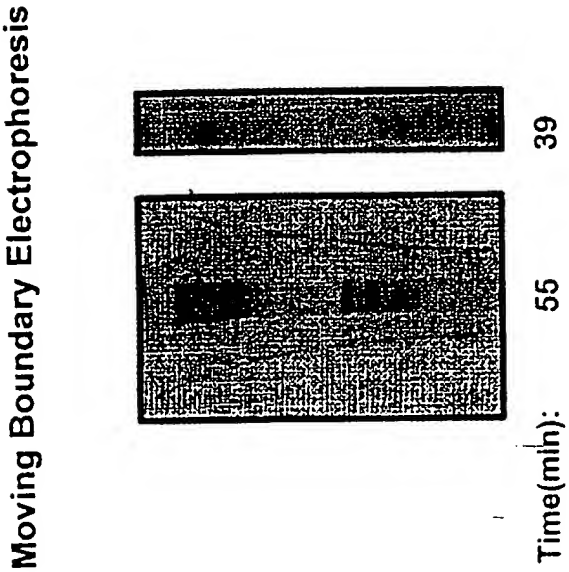

Fig.39

INTERNATIONAL SEARCH REPORT

Int. Ional Application No
PCT/IB 00/00723

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N27/447

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 97 27933 A (HUANG YING ;UNIV TEXAS (US); WANG XIAO BO (US); BECKER FREDERICK F) 7 August 1997 (1997-08-07) figure 2; example 2	1,6
A	EP 0 361 046 A (MJ RESEARCH INC) 4 April 1990 (1990-04-04) abstract; figure 5C	1
A	US 5 569 367 A (BETTS WALTER B ET AL) 29 October 1996 (1996-10-29) abstract; figure 4	1
	-/—	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 July 2000	19/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Zinngrebe, U

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/IB 00/00723

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	US 2 868 316 A (L.V. GUILD) 13 January 1959 (1959-01-13) column 1, line 61-70 column 2, line 56,57	24,28,29
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1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intl ional Application No
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